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CLINICAL ANALYSES

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Steven H. Wong
Medical College of Wisconsin
Milwaukee, Wisconsin

SUPERCRITICAL FLUID CHROMATOGRAPHY FOR THERAPEUTIC DRUG MONITORING OF IMMUNOSUPPRESSANTS: SELECTIVITY FOR CYCLOSPORINE A, FK 506 (TACROLIMUS), AND RAPAMYCIN

STEVEN H. Y. WONG[1]*, BHARTI GHODGAONKAR[1], PETER FONG[1], BRENDAN CAMPBELL[1], JAMES F. BURDICK[2], AND FOUAD BOCTOR[3]

[1]*Department of Pathology*
[2]*Department of Surgery*
The Johns Hopkins University School of Medicine
Baltimore, Maryland
[3]*Department of Laboratory Medicine*
The University of Connecticut School of Medicine
Farmington, Connecticut

ABSTRACT

Clinical monitoring of cyclosporine A (CsA) in whole blood is currently performed by either immunoassays or high-performance liquid chromatography. A new immunosuppressant, FK 506 - Tacrolimus, is currently undergoing multi-centers clinical trial for liver transplant, while active research and clinical studies are being performed for another new immunosuppressant, rapamycin. The present study investigated their chromatographic selectivities by supercritical fluid chromatography, in comparison to HPLC. Feasibility studies were performed for the analyses of extracts of whole blood samples, after solid-phase extraction. SFC analyses were performed by using an open tubular SB-biphenyl capillary column, CO_2 as the mobile phase, pressure programming from 100 to 300 atmospheres, separation temperature of 70° C, and FID detection. CsA eluted after the internal standard CsD, while FK 506 tautomer eluted after FK 506. From the " reversal " of

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*Present address: Department of Pathology, Medical College of Wisconsin, Milwaukee, Wisconsin

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elution order as compared to reversed-phase LC, the selectivity of the above column with CO_2 as the mobile phase was characterized as " normal-phase HPLC-like ". Analysis of extracts of CsA patient's samples, and FK 506 spiked whole blood samples showed that the current SFC procedure did not achieve sufficient sensitivity limit for clinical therapeutic drug monitoring.

INTRODUCTION

Supercritical fluid chromatography(SFC) was advocated for routine pharmaceutical and clinical drug analyses [1]. However, recent reviews by the author indicated limited acceptance as compared to other chromatographic and analytical techniques [2,3]. For pharmaceutical analysis, high-performance liquid chromatography and gas liquid chromatography have provided satisfactory selectivity, capacity, reproducibility, and flexibility, supplemented by GC/MS for definitive analysis. Other forms of analysis include immunoassay and various chemical techniques. For clinical drug analysis, immunoassays have been the methods of choice for high throughput, ready reagent supply, cost effectiveness, and ease of operation, while chromatography, HPLC in particular, has been complementary. For newly introduced drugs such as sertraline [4], an antidepressant, and felbamate [5], an antiepileptics, HPLC provides a readily available and viable alternative. Thus, the role of chromatography for clinical analysis of newly introduced drugs remains firmly established.

In understanding the metabolism of new drugs, the definitive identification and quantitation of both parent drugs, and active and inactive metabolites may be achieved by chromatography, and if needed, interfacing with mass spectrometer for definitive identification. SFC offers similar range in general, and in certain applications, unique advantages, in operational characteristics and chromatographic selectivity as compared to HPLC and GLC [2]. Thus, its clinical application was previously evaluated for therapeutic drug monitoring (TDM) of phenobarbital.[6]

Further, tremendous research and development demonstrated the feasibility of using supercritical fluid extraction(SFE). Messer, Taylor, et al. [7] recently reviewed SFE for drug analysis. To continue our effort in establishing the clinical efficacy, SFC for monitoring immunosuppressants was undertaken.

Currently, several new immunosuppressants are under active clinical investigation for various organ transplants such as liver and kidney. Clinically, CsA has been successfully used for immunosuppression for a variety of organ transplants, while new immunosuppressants, under active clinical investigation but not yet approved by the Federal Drug Administration, would include FK 506 and rapamycin [8]. The current study re-established the " normal-phase HPLC-like " selectivity of SFC for three immunosuppressants - CsA, FK 506, and rapamycin, and described preliminary feasibility extraction studies.

The origin and principle of SFC were previously reviewed [1]. Wong and DellaFera performed SFC analysis of phenobarbital in serum, showing the " normal-phase HPLC-like " selectivity of SFC using CO_2 as the mobile phase [6]. This was important in the design of extraction protocol. Further, solid-phase extraction was advocated in order to introduce " clean " extracts for analysis by open-tubular capillary columns as their sample loading capacity was limited. Li et al. [9] demonstrated the analysis of panaxadiol and panaxatriol in ginseng and Chinese herbal medicine. Biermanns et al. [10] demonstrated SFC chiral analysis of β-blockers such as propranolol. The above applications showed that SFC for drug analysis are still limited for research studies.

First [11] recently projected the future of transplantation. Among issues such as organ availability, further understanding of transplant immunology including tolerance induction, immunosuppressant - both currently used and new ones, had been and will be a vital therapy in preventing organ rejection. New

immunosuppressants, according to a recent review by Napoli, supplementing the currently available CsA, include FK 506 (Tacrolimus) which is undergoing final stage of the multi-center clinical trial for liver transplant, and the beginning stage of renal transplant, cyclosporine G (CsG) - an analog of CsA, rapamycin in limited clinical trials, and other newer agents in various stages of research and clinical studies - mizoribine, mycophenolic acid and its morpholinoethyl ester - RS-61443, brequinar sodium, and deoxyspergualin.   TDM of some of these new immunosuppressants were recently addressed [12]. Figure 1 shows the structures of CsA, FK506 and rapamycin.

The clinical pharmacology and monitoring of CsA have been extensively reviewed and had been the subject of a Task force, review, and Consensus conference [13-15]. From these reports, the mechanism of action of CsA was due to the inhibition of T-cell mediated responses., reduction in interleukin-2(IL-2) synthesis., and inhibition of γ–interferon synthesis. The major side effect of CsA therapy is nephrotoxicity. Monitoring of whole blood CsA is performed clinically by using immunoassays - RIAs, fluorescence polarization immunoassay(FPIA), and enzyme multiplied immunoassay technique., and high performance liquid chromatography using solid-phase extraction and reversed-phase liquid chromatography. According to the latest Consensus conference report [15], the guidelines included specific methods such as selective immunoassays and HPLC to differentiate between CsA and metabolites , and that some of the proposed therapeutic ranges for liver, kidney, bone marrow, and heart tranplants are: 100-150, 80-125, 150-400, and 100-150 µg/L respectively.

Recently, an analog of CsA, CsG underwent clinical trial for renal transplant[16]. Monitoring of CsG was demonstrated by Annesley et al.[16] by using monoclonal RIA and FPIA, and HPLC., and by Yatscoff et al.[17] using selective RIA and FPIA. Their clinical efficacy were acceptable.

Tacrolimus (FK 506), another new immunosuppressant, has been subject of extensive clinical and basic research, and was recently reviewed by Napoli [8], and Wallemacq and Reding [18]. FK 506 is a macrolide isolated from *Streptomyces tsukubaensis*, originally found in Japan [19]. Clinical pharmacology studies showed that FK 506 binds to a cytosolic protein - FK binding protein(FKBP) immunophilin. It inhibits Ca^{2+} dependent division of both T and possibly B cells., inhibits IL-2 and secretion of cytokines., and inhibits the expression of IL-2 receptor. Monitoring of FK 506 was initially performed by ELISA using serum or whole blood [20-23]. More recently, an automated microparticle enzyme immunoassay(MEIA) was introduced by Abbott Laboratory, capable of monitoring whole blood concentrations from 5 to 60 µg/L [24]. For clinical pharmacological research, HPLC and LC/MS have been used to quantify both parent drug and metabolites [25,26].

Rapamycin is another macrolide, isolated from *Streptomyces hygroscopicus* [27-29]. Morris reviewed that rapamycin inhibits both Ca^{2+} dependent and independent pathways in both T and B cells, and IL-2 cannot obviate the inhibition of rapamycin. It blocks T-cell division at the G1/S phase, different from that of FK 506 at the G0/G1 phase. It lowers internalization of IL-2. Similar to FK 506, it binds to FKBP immunophilin. Napoli and Kahan [30] optimized an HPLC analysis by using two microbore Novapak columns(150 mm x 2.1 mm), water/methanol(1:9), 0.1 mL/min., and 40° C. Retention time was 12 min. Yatscoff, Faraci and Bolingbroke [31] recently described an HPLC assay of rapamycin, using an ether extraction, followed by a reversed-phase analysis using two Spherisorb C-8 columns in tandem, methanol/water as the mobile phase, 0.35 mL/min, 45° C, and detection at 278 nm. Linearity was established for 250 µg/L, with a sensitivity limit of 1 µg/L. From the animal model study using rabbits and

cyclosporine A
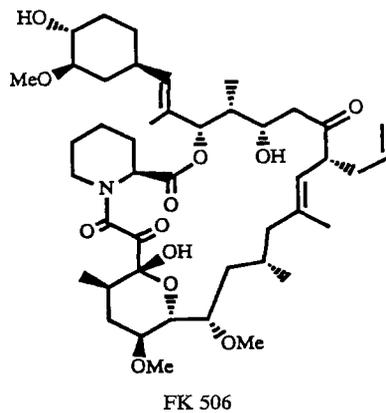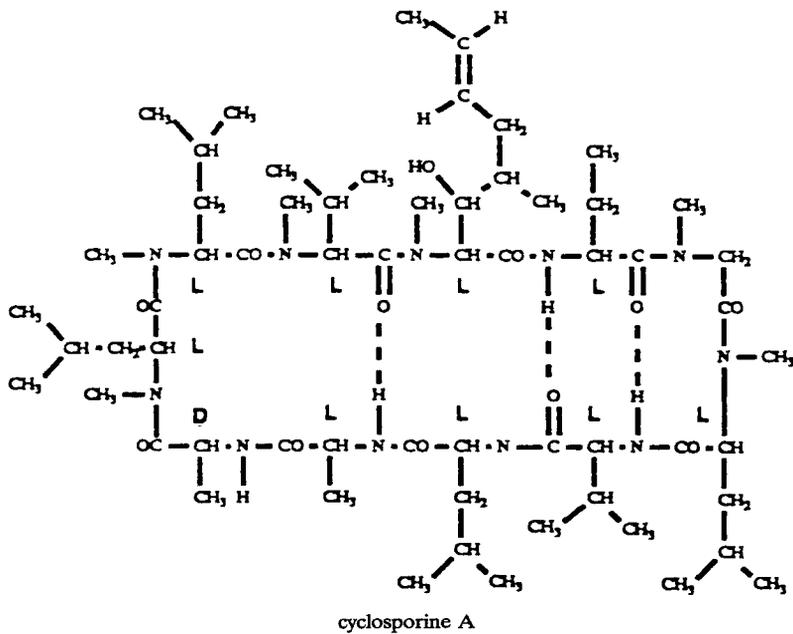
FK 506

Figure 1: Structures of cyclosporine A, FK 506 and rapamycin.
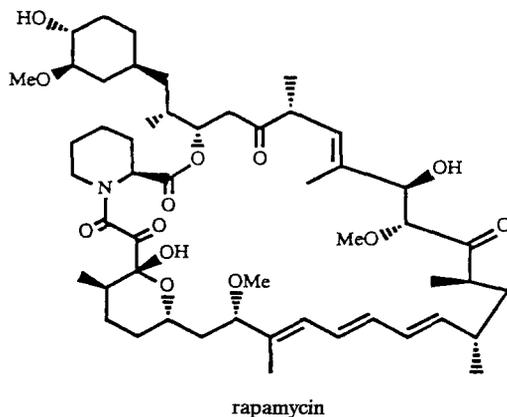
rapamycin

FIGURE 1 (continued)

from other pharmacokinetic studies, a trough concentration of about 0.75 to 30 μg/L was proposed.

The current studies investigated the SFC selectivity of CsA, FK 506 and rapamcyin, and some feasibility studies were performed for CsA and FK 506 whole blood extracts [32].

Experimentals

I. Reagents - Hexane, resi-analyzed grade, acetonitrile, methanol, HPLC grade, and zinc sulphate were obtained from J.T. Baker (Phillipsburg, NJ). Bond-Elut C-18 extraction cartridges were obtained from Varian (Walnut Creek, CA). Supercritical grade carbon dioxide was provided by Scott Specialty Gases (Plumsteadville, PA).

II. Drug standard solutions - Cyclosporine A and D were kindly donated by Sandoz Pharmaceuticals(Hanover, NJ). FK 506 (Tacrolimus) was generously supplied by Fujisawa Pharmaceutical Company (Deerfield, IL). Rapamycin was

donated by Wyeth-Ayerst Research (Princeton, NJ). To prepare the primary drug

standard solutions of 1 mg/mL, 10 mg of each of the above drugs was dissolved in

10 mL of methanol. In preparing the working stock solutions, 100 µL was further

diluted with 10 mL of methanol, yielding a final concentration of 10 ng/µL.

Primary stock solutions were injected into both SFC and HPLC for selectivity

studies, while the working stock solutions were used for spiking into drug-free

whole blood for extraction feasibility studies.

      III. Instrumentations - Two SFC were used for the present study. For the

selectivity and feasibility studies of CsA, the SFC was consisted of a Model 500

SFC pump from Dionex/Lee Scientific (Salt Lake City, Ut). A model 600 controller

was used to execute the pressure programming. Supercritical fluid was delivered to

a Valco injector with a 200 nL loop, mounted on top of a model 5880 gas

chromatograph equipped with a FID detector from Hewlett Packard (Avondale,

PA). An open tubular capillary column was used, SB-biphenyl-100, 10 m in length

and 50 µm internal diameter, obtained from Dionex/Lee Scientific Division (Salt

Lake City, Ut). A Hewlett Packard monitor was used for recording key

chromatographic parameters and the signal from the FID. The SFC used for the

analyses of FK 506 and rapamycin was a Model 600 series from Dionex/Lee

Scientific. HPLC analysis was performed by a Model 9533 liquid chromatograph,

equipped with a Model 9523 variable wavelength detector from IBM instrument(

Danbury, CT), and a reversed-phase µBondapak C-18 column(20 cm x 3.6 mm),

Waters/Millipore(Milford, MA).

      IV. Sample preparation - Drug-free whole blood samples, 1 to 4 mL were

spiked with the working stock solutions. Along with quality control and selected

patient samples, a solid-phase extraction was performed using a Bond-Elut C-18

cartridge. Aliquots, 1 to 4 mL of the above whole blood samples were vortex-

mixed with zinc sulfate/methanol/acetonitrile for protein precipitation. After

centrifugation, the supernatant was transferred to a series of Bond-Elut C-18 extraction cartridges, pre-conditioned by elution with methanol and water. After elution of the supernatant, the column was washed with acetonitrile and water(9:11), followed by water. The cartridges were dried for 30 minutes under vacuum. Then, the extracted drugs were eluted with methanol. Following concentrating the extracts by evaporating the methanol, the residues were re-constituted with methanol for SFC analysis. For HPLC analysis, methanolic extracts were mixed with water, followed by washing with n-hexane. Aliquots of the transferred aqueous layer was injected into the HPLC for analysis.

V. Chromatographic conditions - SFC parameters were: SB-biphenyl column, CO_2 as the mobile phase, 70° C, 100 to 300 atmosphere at 10 atmos/min for the three drugs, and 175 to 250 atmosphere at 2 atmos/min for CsA and CsD, splitted injection of about 200 nL injected, and FID detection. HPLC parameters were: µBondapak C-18, acetonitrile/water at 1 mL/min, 70° C, injection volume of 50 to 100 µL, and detection at 202 nm at 0.002 AUFS.

Results and discussions

Based on the previous study of SFC analysis of phenobarbital in serum [6], a solid phase extraction protocol was used for the whole blood extraction of CsA. Figure 2 shows the SFC chromatogram of an extract of a patient's whole blood with about 228 µg/L of CsA. Pressure programming was used, with initial 100 and final 300 atmosphere as stated previously. CsA eluted at about 18.4 minutes, equivalent to the elution pressure of 270 atmosphere. Unfortunately, CsA co-eluted with the internal standard CsD. Thus, this would not useful for quantitative application. However, this data was useful to guide further optimization study.

In order to achieve the necessary resolution, the next approach was " fine-tuning " the pressure programming. After systematic studies, the initial and final
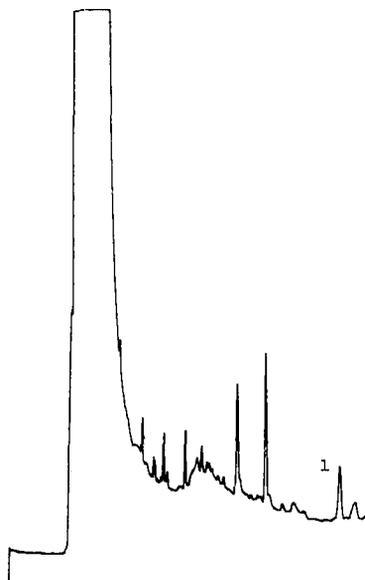
Figure 2: SFC chromatogram of a whole blood extract of a bone marrow tranplant patient with about 228 µg/L of CsA at 18.4 min. Pressure programming from 100 to 300 atmosphere at 10 atmos/min. Peak identification: 1, CsA.

pressures were chosen to be 175 and 250 atmosphere, with a " mini-step " pressure programming of 2 atmos/min. Figure 3 shows the successful resolution of CsA, peak 1 at about 35 min, after CsD the internal standard as peak 2 at 33 minutes. The extraction was performed with 4 mL of whole blood samples. The estimated CsA concentration was about 220 µg/L of whole blood from a bone marrow transplant patient. Since the therapeutic range of CsA for bone marrow transplant was proposed to be 150 to 400 µg/L,[13] this concentration represented a therapeutic concentration. Even though 4 mL of samples was used, both the apparently poor extraction recovery and poor FID response rendered this quantitation to be
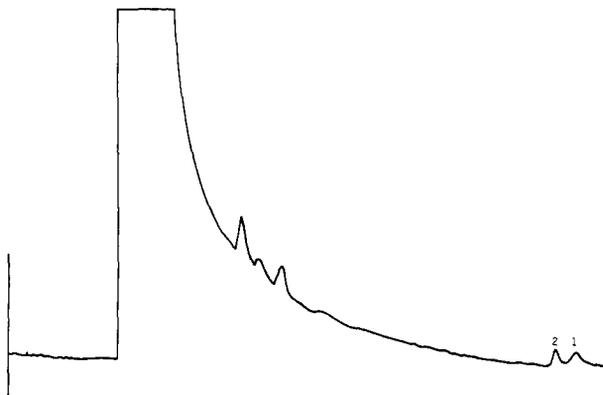
Figure 3: SFC chromatogram of a whole blood extract of a bone marrow transplant patient with about 220 μg/L of CsA . Pressure programming from 175 to 250 atmosphere at 2 atmos/min. Peak identification: 1. CsA   at 35 min., and 2.CsD(Internal standard) at 33 min.

unacceptable. Sensitivity may be enhanced by using packed SFC column for increased loading capacity, other detectors with higher sensitivity, or by interfacing with mass spectrometer in the future.

However, from this study, the reversal of elution order of the " more polar " CsA and the " less polar " CsD in relation to RPLC confirmed the key observation on " normal-phase HPLC-like " selectivity of carbon dioxide and SB-biphenyl column as seen previously. This was also observed in the selectivity for analysis of FK 506 and its tautomer.

Figure 4A shows the SFC chromatogram of FK 506 drug standard solution as peak 1, and its tautomer, peak 2 with retention times of 17.5 and 17.8 minutes. The reversed order is shown in the HPLC chromatogram Figure 4B, FK 506 eluting after its tautomer at 5 and 4 minutes respectively. This elution order was also previously established by Friob et al [25]. Preliminary studies indicated serum
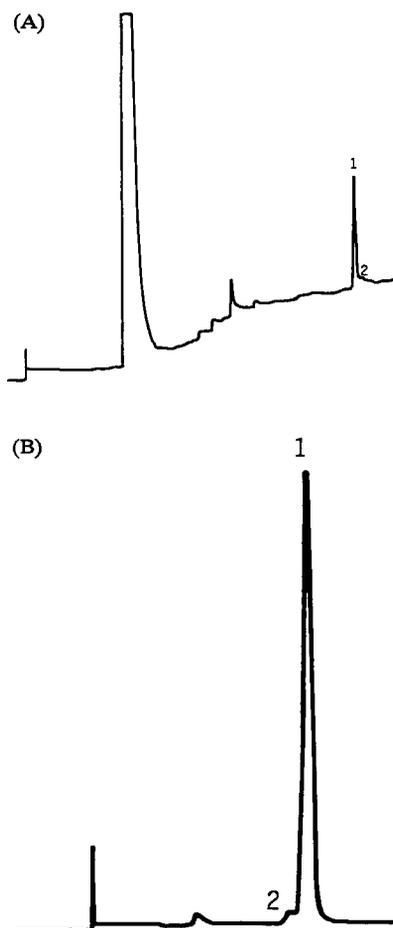
Figure 4: (A) SFC, and (B) HPLC chromatograms of FK 506. Peak identification: 1, FK 506; 2, FK 506 tautomer.
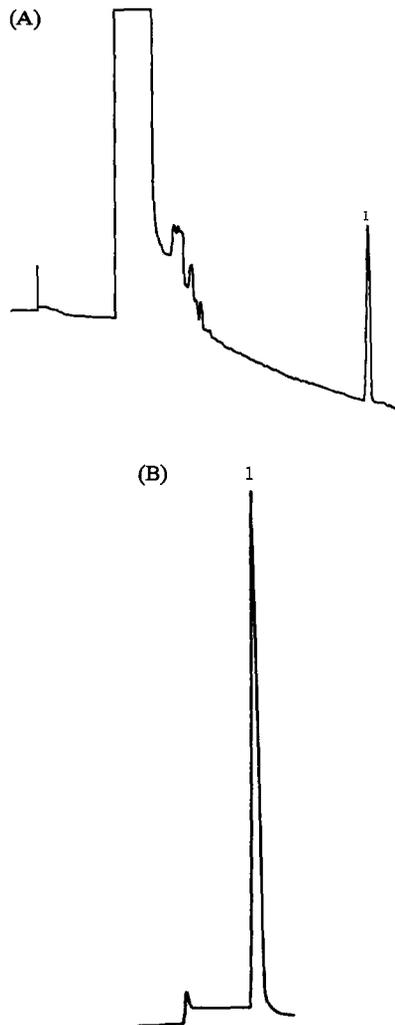
Figure 5: (A) SFC, and (B) HPLC chromatograms of rapamycin.

concentration of about 0.5 to 5 µg/L and whole blood concentrations of 5 to 20 µg/L [18]. Whole blood spiked at 20 µg/L did not reveal detectable signal. This was logical based on the previous experience with the CsA analysis of using open tubular capillary column.

In establishing the SFC selectivity of rapamycin, primary drug standard was also analyzed with a similar pressure program to FK 506. Figure 5 A and B show the SFC and RPLC chromatograms with retention times of 18.4 and 5 minutes respectively. Similar disappointing result was obtained from the analysis of whole blood samples spiked with rapamycin.

Conclusion

The present study established the selectivity of SFC analysis of immunosuppressants to be " normal -phase HPLC-like ". This selectivity was important in understanding the elution order and the design of the extraction protocol. The low extraction recovery and low sensitivity of the FID did not allow adequate quantitation of these drugs in whole blood, and these may be obviated by using packed SFC column, and high sensitivity techniques such as mass spectrometer in the future.

References

1. Wong SHY. Supercritical fluid and microbore liquid chromatography for clinical drug analysis. Clin Chem , 1989; 35:1293-1298.

2. Wong, SHY. Novel liquid chromatographic techniques for clinical drug analysis. Therapeutic drug monitoring and toxicology. Amer Assoc Clin Chem In-Service Training & Continuing Education 1992;13:5-24.

3. Wong, SHY. Advances in chromatography for clinical drug analysis: Supercritical fluid chromatography, capillary electrophoresis, and selected high-performance liquid chromatography techniques. Ther Drug Monit.1993;15:576-580.

4. Wong SHY, Gulamali-Majid F, Campbell BT, Fong P, Hoehn-Saric R, Wisson LS, Kranzler HR, DellaFera S, Fernandes R. Sertraline, N-desmethyl sertraline, and fluvoxamine monitoring by reversed-phase liquid chromatography. Clin Chem. 1993;39:1244-1245. (Abstract).

5. Remmel RP, Miller SA, Graves NM. Simultaneous assay of felbamate plus carbamazepine, phenytoin, and their metabolites by liquid chromatography with mobile phase optimization. Ther Drug Monit. 1990;12:90-96.

6. Wong SHY, DellaFera SS. Supercritical fluid chromatography for therapeutic drug monitoring and toxicology: Methodological consideration for open capillary tubular column for the analysis of phenobarbital. J Liq Chromatogr Clin Anal. 1990;13:1105-1124.

7. Messer DC, Taylor LT, Moore WN, Weiser WE. Assessment of supercritical fluids for drug analysis. Ther Drug Monit. 1993;15:581-587.

8. Napoli KL. Immunosuppressive agents for the 1990s. J Int Fed Clin Chem. 1991; 4:15-23.

9. Li YH, Li XL, Hong I, Liu JY, Zhang MY. Determination of panaxadiol and panaxatriol in ginseng and its preparations by capillary supercritical fluid chromatography (SFC). Biomed Chromatogr 1992;6:88-90.

10. Biermanns P, Miller C, Lyon V, Wilson W. Chiral resolution of β-blockers by packed-column supercritical fluid chromatography. LC-GC. 1993;11:744-747.

11. First MR. Transplantation in the Nineties. Transplantation. 1992;53:1-11.

12. Yatscoff RW, Shaw LM. Therapeutic monitoring of cyclosporine, FK 506, and rapamycin. Ther Drug Monit 1992;14:267.

13. Shaw LM, Demers L, Freeman O, et al. Critical issues in cyclosporine monitoring: Report of task force on cyclosporine monitoring. Clin Chem. 1987;33:1269-1288.

14. Kahan BD. Cyclosporine. NEJM 1989;321:1725-1738.

15. Shaw LM, Yatscoff RW, Bowers LD, et al. Canadian consensus meeting on cyclosporine monitoring: Report of the Consensus panel. Clin Chem. 1990;36:1841-1846.

16. Annesley TM, Coombs RC, Orsulak PJ. Comparison of cyclosporin G (Nva^2-Cyclosporin) concentrations measured in whole blood by monoclonal fluorescence polarization immunoassay, monoclonal radioimmunoassay, and HPLC. Clin Chem. 1993;39:1050-1053.

17. Yatscoff RW, Langman LJ, LeGatt DG. Cross-reactivities of cyclosporin G (Nva^2-Cyclosporin) and metabolites in cyclosporin A immunoassays. Clin Chem. 1993;39:1089-1092.

18. Wallemacq PE, Reding. FK 506 (Tacrolimus), a novel immunosuppressant in organ transplantation: Clinical, biomedical, and analytical aspects. Clin Chem; 1993:11:2219-2228.

19. Kino T, Hatanaka H, Hashimioto M, et al. FK 506, a novel immunosuppressant isolated from a streptomyces. I. Fermentation, isolation and physico-chemical and biological characteristics. J Antibiot 1987;40:1249-1255.

20. Kobayashi M, Tamura K, Katayama N, et al. FK 506 assay - past and present - characteristics of FK 506 ELISA. Transplant Proc.1991;23:2725-2729.

21. Wallemacq PE, Firdaous I, Hassoun A. Improvement and assessment of enzyme-linked immunosorbent assay to detect low FK 506 concentrations in plasma or whole blood within 6 hours. Clin Chem. 1993;39:1045-1049.

22. Jusko WJ, D'Ambrosio. Monitoring FK 506 concentrations in plasma and whole blood. Transplant Proc. 1991;23:2732-2735.

23. Warty VS, Venkataramanan R, Zendehrough P et al. Practical aspects of FK 506 analysis(Pittsburgh experience). Transplant Proc. 1991;23:2730-2731.

24. Grenier FC, Luczkiw J, Bergmann M, et al. A whole blood FK 506 assay for the IMx Analyzer. Transplant Proc. 1991;23:2748-2749.

25. Friob MC, Hassoun A, Latinne D, Lhoest G, Otte JB, Wallemacq PE. A combined HPLC-ELISA evaluation of FK 506 in transplant patients. Transplant Proced 1991;23:2750-2752.

26. Christians U, Braun F, Kosian N, et al. High performance liquid chromatography/mass spectrometry of FK 506 and its metabolites in blood, bile and urine of liver grafted patients. Transplant Proc. 1991;23:2741-2744.

27. Sehgal SN, Baker H, Vezina C. Rapamycin (Y-22,989) a new antifungal antibiotic II. Fermentation, isolation and characterization. J Antibiot. 1975;28:727-733.

28. Kahan BD, Chang J. Sehgal SN. Preclinical evaluation of a new potent immunosuppressive agent, Rapamycin. Transplantation 1991;52:185-191.

29. Morris RE. Rapamycins: Antifungal, antitumour, antiproliferative and immunosuppressive macrolides. Transplant Rev 1992;6:39-87.

30. Napoli KL, Kahan BD. High-pressure liquid chromatography of rapamycin. Clin Chem. 1991;37:294-295.

31. Yatscoff RW, Faraci C, and Bolingbroke P. Measurement of rapamycin in whole blood using reverse-phase high-performance liquid chromatography. Ther Drug Monit. 1992;14:138-141.

32. Wong SHY, Ghodgaonkar B, Fong P, Burdick JF, Boctor F. Supercritical fluid chromatography and HPLC of cyclosporine and FK-506. Clin Chem. 1992;38:995-996. (Abstract)

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF HYDROGEN PEROXIDE WITH PEROXYOXALATE CHEMILUMINESCENCE DETECTION

KENICHIRO NAKASHIMA[1], MITSUHIRO WADA[1], NAOTAKA KURODA[1], SHUZO AKIYAMA[1], AND KAZUHIRO IMAI[2]

[1]School of Pharmaceutical Sciences
Nagasaki University
1-14 Bunkyo-machi, Nagasaki 852, Japan
[2]Branch Hospital Pharmacy
University of Tokyo
3-28-6 Mejirodai, Bunkyo-ku, Tokyo 112, Japan

ABSTRACT

A high performance liquid chromatographic method with peroxyoxalate chemiluminescence (PO-CL) detection was developed for the determination of hydrogen peroxide. Hydrogen peroxide separated by a reversed phase column was detected with the CL produced by the PO-CL reaction using a mixture of 2,4,6,8-tetrathiomorpholinopyrimido[5,4-d]pyrimidine and bis[2-(3,6,9-trioxadecyloxycarbonyl)-4-nitrophenyl]oxalate or bis(2,4,6-trichlorophenyl)oxalate in acetonitrile as a post column reagent. Hydrogen peroxide could be determined over the range of 5-100 pmol on column and the detection limit was 188 fmol at a signal-to-noise ratio of 3. Relative standard deviation of 10-replicate measurements of peak heights for 50 pmol was 2.36%. The detection system applied to the assay of hydrogen peroxide in cola drinks. Trace amounts of hydrogen peroxide could be determined in six cola drinks. The levels were 68.4±2.6-174.2±8.4 nmol/dl. Hydrogen

peroxide contaminated in commercially available hydro-
peroxides was also determined by the proposed method.
The amounts obtained were 0.061% for t-butyl hydroper-
oxide and 0.003% for cumene hydroperoxide.

INTRODUCTION

It is well known that hydrogen peroxide can be
enzymatically generated by the reaction of various
oxidases. Therefore, sensitive and selective methods
for the determination of hydrogen peroxide have been
requisite for the field of biological or biomedical
chemistry. For this purpose, a peroxyoxalate chemilumi-
nescence (PO-CL) detection system is very suitable
because of its highly selectivity and sensitivity to
hydrogen peroxide [1-5]. Recently, we have developed
new fluorescent components for PO-CL, i.e., pyrimido-
[5,4-d]pyrimidine derivatives, and utilized for the
sensitive determination of hydrogen peroxide and glu-
cose by a photographic technique [3]. By this PO-CL
system, glucose and uric acid in serum also have been
sensitively determined using a flow injection method
[4]. On the other hand, to our knowledge, a high per-
formance liquid chromatographic (HPLC) method with CL
detection for the assay of hydrogen peroxide has not
been developed. In this paper, thus, we tried to
develop a HPLC method with PO-CL detection to analyze

hydrogen peroxide using a mixture of 2,4,6,8-tetrathio-
morpholinopyrimido[5,4-d]pyrimidine   (TMP)   and   bis-
(2,4,6-trichlorophenyl)oxalate (TCPO) or bis[2-(3,6,9-
trioxadecyloxycarbonyl)-4-nitrophenyl]oxalate (TDPO) in
acetonitrile as a post column CL reagent. Then, we ap-
plied the method to the assay of hydrogen peroxide in
ascorbic acid, cola drinks and some commercially avail-
able peroxides.

MATERIALS

     TMP   was synthesized as described previously and
recrystallized   from   chloroform-methanol   [6].   TDPO,
hydrogen peroxide (30%), ascorbic acid, and m-chloro-
perbenzoic acid were obtained from Wako Pure Chemical
Co. (Osaka, Japan). TCPO and imidazole were   purchased
from   Tokyo   Kasei Kogyo Ltd. (Tokyo,   Japan).   t-Butyl
hydroperoxide (Katayama Chemical Co.,   Tokyo,   Japan),
cumene hydroperoxide (Nacalai tesque,   Tokyo,   Japan),
and catalase (6500 U/ml, Bheringer Manheim Yamanouchi,
Tokyo, Japan) were used as obtained. All other reagents
used were analytical grade. Acetonitrile used   HPLC
grade (Wako). Water was deionized and further purified
by a WL 21P purifying system (Yamato, Tokyo, Japan).
     Buffer solutions were prepared as follows: (1) 10
(or 100) mM imidazole buffer (pH 7.0); imidazole (0.68

g for 10 mM, 6.80 g for 100 mM) was dissolved in 800 ml H_2O, adjusted the pH at 7.0 with nitric acid, and diluted to 1000 ml with water, (2) 0.5 M phosphate buffer (pH 7.0); 0.5 M KH_2PO_4 was mixed with 0.5 M Na_2HPO_4 to adjust the pH at 7.0.

METHODS

HPLC Apparatus

The flow system used is shown in Fig. 1. The HPLC system consisted of two LC 6A HPLC pumps (Shimadzu, Kyoto, Japan), a 7125 injector (Rheodyne, Cotati, CA, USA) with 20 μl sample loop, a separation column [STR ODS-II (150 x 4.6 mm, I.D., 5 μm, Shimadzu), CHEMCOSORB 5-ODS-UH (150 x 4.6 mm, I.D., 5 μm, Chemco, Tokyo, Japan) and TSK ODS-80TM (250 x 4.6 mm, I.D., 5 μm, Tosoh, Tokyo, Japan), a detector [825-CL and UVI-DEC-100-1V, Jasco, Tokyo, Japan], a SC-77 signal clean-er (Sic, Tokyo, Japan), and a FBR-1 recorder (Tosoh). For the measurement of three dimensional chromatograms, a Shimadzu LC-9A HPLC system equipped with SPD-M6A photodiode array UV-VIS detector was used.

HPLC conditions

The PO-CL reaction conditions for the determina-tion of hydrogen peroxide were examined by a STR ODS-II
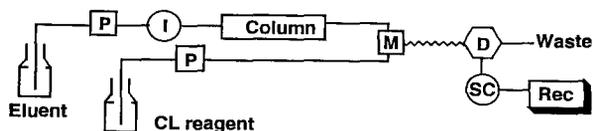
FIGURE 1. Flow system for HPLC-CL detection.

P: pump; I: injector; M: mixing tee; D: detector; SC: signal cleaner; Rec: recorder.

reversed phase column with an eluent of 10 mM imidazole buffer(pH 7.0)-acetonitrile (90/10, v/v) at a flow rate of 1 ml/min. A mixture of TMP and TDPO in acetonitrile was used as a post column reagent at a flow rate of 1 ml/min. Hydrogen peroxide in cola drinks and ascorbic acid was separated with TSKgel ODS-80TM with an eluent of 10 mM imidazole buffer (pH 7.0)-acetonitrile (60/40, v/v) at a flow rate of 1 ml/min. A post column reagent used was a mixture of TMP and TDPO in acetonitrile at a flow rate of 1 ml/min. For the separation of hydrogen peroxide from other peroxides, CHEMCOSORB 5-ODS-UH was used with an eluent of 10 mM imidazole buffer (pH 7.0)-methanol (30/70, v/v) at a flow rate of 0.8 ml/min. A mixture of TMP and TCPO in acetonitrile was used as a post column reagent at a flow rate of 1 ml/min.

Measurement of Time Course for the Consumption of Hydrogen Peroxide with Catalase

To a sample solution (700 μl) were added 300 μl 0.5 M phosphate buffer (pH 7.0) and 10 μl catalase

(6500   U/ml),   and incubated at 37 °C for 90   min.   At
every   30   min, a portion (200 µl) of the   mixture   was
pipetted,   passed through a membrane filter (0.45   µm),
and the filtrate was injected onto HPLC.

Measurement of the Stability of the Post Column Reagent

     TCPO-TMP: a   mixture of TCPO (0.3   mM)   and   TMP
$(5x10^{-6}$ M) in acetonitrile was kept in an amber   bottle
at   room temperature. A 20 µl portions of   the   mixture
was   injected onto HPLC at a definite   interval.   TCPO,
TMP and a decomposed product of TCPO   were separated on
STR-ODS-II column using a mobile phase of acetonitrile-
water   (95/15,   v/v) at a flow rate of 1.0   ml/ml,   and
monitored at 280 nm.

     TDPO-TMP: a   mixture   of TDPO (0.3   mM)   and   TMP
$(5x10^{-6}$ M) in acetonitrile was kept in an amber   bottle
at   room temperature. A 20 µl portions of   the   mixture
was   injected onto HPLC at a definite   interval.   TDPO,
TMP and a decomposed product of TDPO   separated   on
STR-ODS-II column with a mobile phase of   acetonitrile-
water   (99/1,   v/v) at a flow rate of 1.0   ml/min,   and
monitored at 310 nm.

Availability of the Post Column Reagent

     Twenty µl of H_2O_2 (containing 50 or 100 pmol)   was
injected   on to HPLC at a definite interval,   separated

on STR-ODS-II column with a mobile phase of 100 mM imidazole buffer (pH 7.0)-acetonitrile (60/40, v/v) at a flow rate of 1 ml/min, reacted with a post column reagent of TCPO (0.3 mM) and TMP (5×10^{-7} M) in acetonitrile or TDPO (0.3 mM) and TMP (2.5×10^{-7} M) in acetonitrile, and the resulted CL was monitored.

RESULTS AND DISCUSSION

Post Column PO-CL Reagent

In the previous study, we used a mixture of TCPO and TMP in acetonitrile as the post column reagent for the flow injection analysis of hydrogen peroxide derived from glucose or uric acid by the enzymatic reaction with immobilized enzyme reactor [3]. In this case, three delivery pumps were used for a carrier solvent, TCPO and TMP solutions. Some research works on PO-CL have revealed that a mixture of hydrogen peroxide and TCPO (or TDPO) could be used as a PO-CL reagent for the determination of fluorophores, and consequently, one of the two delivery pumps for reagents could be reduced [5-7]. On the other hand, as the post column reagent, a mixture of fluorescent compound and aryloxalate in acetonitrile seemed to be applicable for the determination of hydrogen peroxide. Thus we studied first the

use a mixture of fluorescent compound (TMP) and TCPO (or TDPO) in acetonitrile as a post column PO-CL reagent. First, the stability of the reagent mixture was examined. A HPLC method with UV detection was used for the measurement of time course of the relative peak heights for TCPO (or TDPO) and TMP in acetonitrile. The chromatograms obtained were shown in Fig. 2. As shown in Fig. 3, the relative peak heights for TCPO (or TDPO) alone were stable at least for 24 h. TCPO in the mixture with TMP was also stable, but TDPO was less stable compared to TCPO and decomposed gradually. TMP itself in the mixture with TCPO or TDPO was less stable than oxalates coexisted.

The availability of a mixture of TMP and TCPO or TDPO in acetonitrile as the post column reagent was measured as a function of relative CL intensity produced from the reaction with a known concentration of hydrogen peroxide. The time course of CL intensity for hydrogen peroxide (100 pmol/injection) was shown in Fig. 4. The relative CL intensity obtained with TMP-TDPO reagent was ca. 1.5 times larger than that with TMP-TCPO reagent and both CL intensities were almost constant at least for 8h. These results show that a mixture of TMP and TCPO or TDPO in acetonitrile could be used as a post column reagent within 8h under the conditions used.
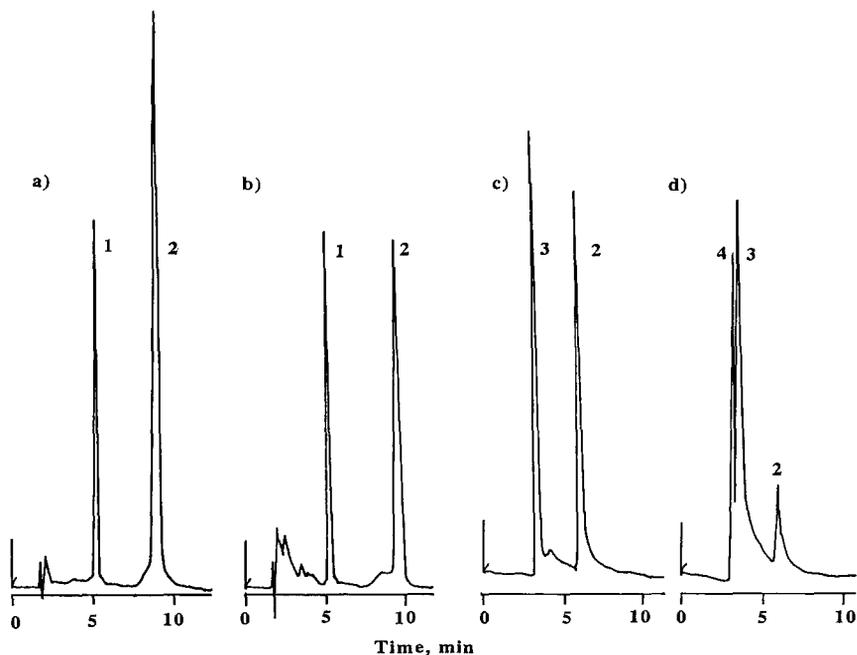
FIGURE 2. Chromatograms for TCPO-TMP and TDPO-TMP solutions in acetonitrile.

a); TCPO-TMP at 0h, b); TCPO-TMP at 24h after; c); TDPO-TMP at 0 h, d); TDPO-TMP at 24 h after; Peaks: 1; TCPO, 2; TMP, 3; TDPO, 4; Decomposed product. Other experimental conditions are described in the text.

Determination of Hydrogen Peroxide

A HPLC-CL determination of hydrogen peroxide was examined using STR-ODS-II and a mixture of TDPO and TMP in acetonitrile as an analytical column and a post column CL reagent, respectively. Hydrogen peroxide was separated at a retention time of ca. 2 min with a
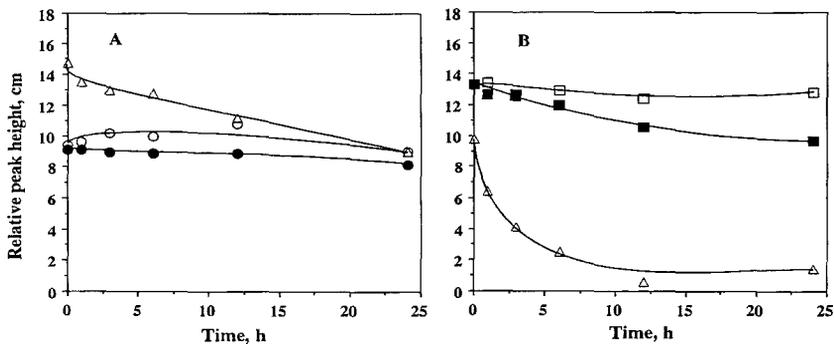
FIGURE 3. Stabilities of the post column CL reagents.

A: (○)TCPO, (●)TCPO in TCPO-TMP, (△) TMP in TCPO-TMP; B: (□)TDPO, (■)TDPO in TDPO-TMP, (△)TMP in TDPO-TMP. Other experimental conditions are described in the text.
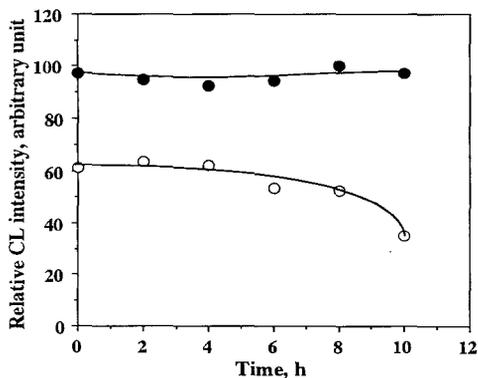
FIGURE 4. Availabilities of the post column CL reagents.

Sample: H_2O_2, 100 pmol/injection); TDPO-TMP (●); TCPO-TMP (○). Other experimental conditions are described in the text.

mobile phase of 10 mM imidazole buffer (pH 7.0)-CH_3CN

(90/10, v/v) at a flow rate of 1 ml/min. Optimization

of the CL reagent concentration was examined with

hydrogen peroxide (50 pmol/injection). Though   the

relative CL intensity was increased with an increase of

the concentration TDPO and TMP, the back-ground level

was also increased; a 1:1 mixture of 0.6 mM TDPO in

acetonitrile and 5×10^{-7} M TMP in acetonitrile gave the

largest S/N ratio, which was used as a post column

reagent. In this work, we studied the effect of a

signal cleaner (SC77, SIC) on chromatograms and found

that it increased the S/N ratio more than 5 times. The

calibration curve for H_2O_2 was linear over the range

from 5 to 100 pmol (r=0.999), and the detection limit

was 188 fmol (9.9×10^{-8} M, S/N=3). The relative standard

deviation of peak heights for 10-replicate measurements

of 50 pmol H_2O_2 was 2.36%. The sensitivity of this

method is higher than that of our previous flow-

injection analysis (FIA) with CL detection method

(5×10^{-8} M)[4] and comparable to that of PO-CL method

using immobilized fluorophores (1×10^{-8} M)[1], but

slightly lower than those of PO-CL detection with

fiber-optic sensor (2.5×10^{-9} M)[2] and FIA-PO-CL method

using sulforhodamine 101 as a fluorescent component

(3×10^{-9} M, S/N=2)[5].

Determination of Hydrogen Peroxide in Ascorbic Acid and Cola Drinks

Trace amounts of hydrogen peroxide have been found in drinks containing ascorbic acid or caramel. Therefore, a quantitative determination of H_2O_2 contaminated in commercially available ascorbic acid and cola drinks was studied using ODS-80TM and a mixture of TDPO and TMP in acetonitrile as an analytical column and a post column CL reagent, respectively. The eluent used was 10 mM imidazole buffer/CH_3CN(60/40, v/v). Retention times for ascorbic acid and H_2O_2 were confirmed by a three dimensional UV detection. Separation of H_2O_2 spiked in cola drink was also achieved by the same conditions (Fig. 5). The peak corresponded to H_2O_2 in ascorbic acid and cola was disappeared with treatment of catalase (Fig. 6). Figure 7 shows typical chromatograms for CL detection of H_2O_2 in ascorbic acid and cola drink. Contents of hydrogen peroxide determined in two commercially available ascorbic acids were 1.49 ± 0.01%(n=4) and 1.30 ± 0.01%(n=4). Several kinds of cola drinks were assayed and 68.4 - 174.2 nmol/dl of H_2O_2 could be determined. These results are briefly summarized in Table 1.

Determination of Other Peroxides

As peroxides have been known to cause serious damages for living cells, it is biologically important
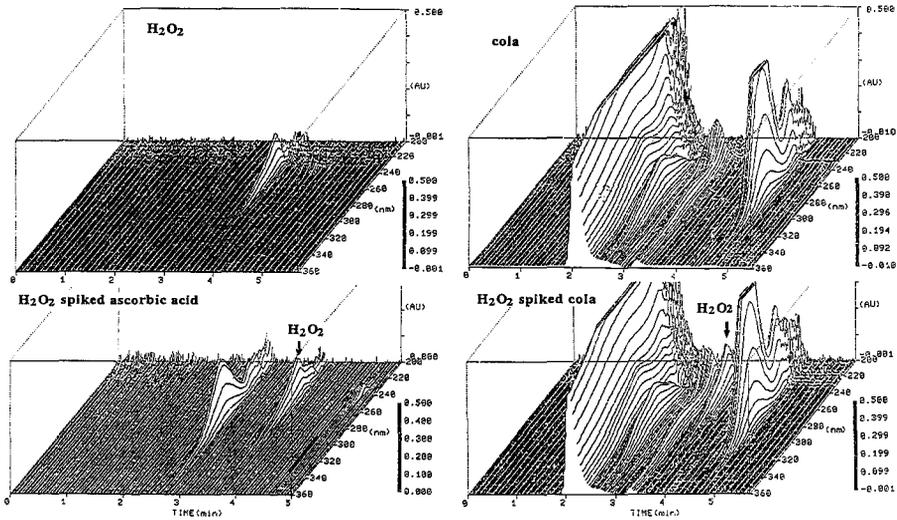
FIGURE 5. Chromatograms with three dimensional UV detection for H_2O_2, H_2O_2 spiked ascorbic acid and H_2O_2 spiked cola.

Sample: H_2O_2; $1x10^{-2}$ M.
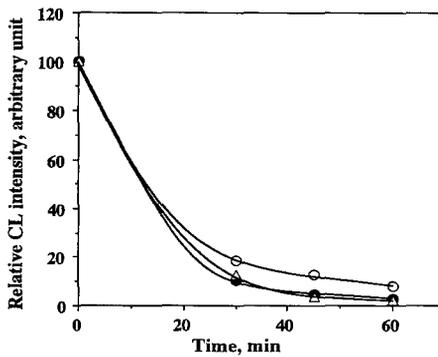
FIGURE 6. Time course for catalase reaction with H_2O_2.

Sample: ascorbic acid; $1x10^{-4}$ M, H_2O_2; $5x10^{-7}$ M, cola; undiluted.
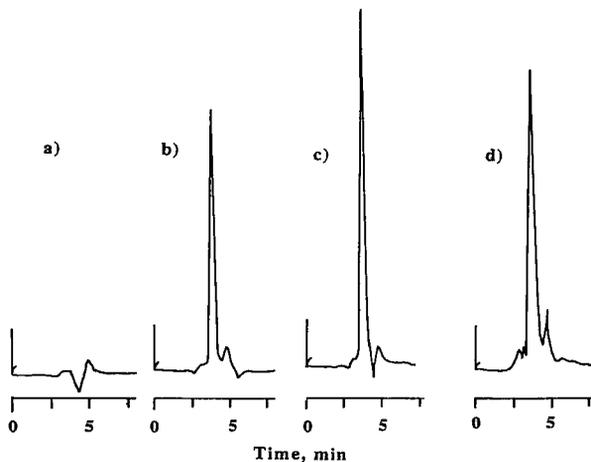
FIGURE 7. Chromatograms for H_2O_2 and H_2O_2 in ascorbic acid and cola drink.

a); blank (H_2O), b); H_2O_2 (1×10^{-6} M), c); ascorbic acid (2.5×10^{-5} M), d); cola (undiluted). Other experimental conditions are described in the text.

TABLE 1. Amounts of Hydrogen Peroxide in Cola Drinks

Sample	nmol/dl	RSD%, (n)
cola A	174.2 ± 8.4	5.54 (10)
cola B	92.5 ± 7.1	7.54 (10)
cola C	114.5 ± 3.8	3.72 (10)
light cola A	85.4 ± 1.9	2.22 (5)
diet cola A	68.4 ± 2.6	3.80 (5)
diet cola B	74.2 ± 2.9	3.84 (5)

to develop sensitive methods to determine a trace amount of them. It is obvious that peroxides can be detected by the PO-CL detection method. Thus we studied the determination of some commercially available peroxides, i.e., t-butyl hydroperoxide, cumene hydroperoxide, and m-chloroperbenzoic acid. For HPLC conditions, a CHEMCOSORB 5-ODS-UH column and a mixture of TCPO and TMP in acetonitrile were used as an analytical column and a CL reagent, respectively. Separation was done with an eluent of 10 mM imidazole buffer (pH7)/MeOH(30/70, v/v). Retention time for H_2O_2, t-butyl hydroperoxide, cumene hydroperoxide and m-chloroperbenzoic acid were 1.9, 3.1, 5.0 and 5.0 min, respectively. In the course of the experiment, we found that H_2O_2 was contaminated in hydroperoxides. Hydrogen peroxide concentrations contaminated in t-butyl hydroperoxide and cumene hydroperoxide were 0.061% and 0.003%, respectively, but could not be detected in m-chloroperbenzoic acid. The relative CL intensities for t-butyl hydroperoxide and cumene hydroperoxide were 0.47 and 0.26 when that of H_2O_2 was taken as 100. This result means that H_2O_2 gave very strong CL compared to other peroxides and, therefore, suitable for oxidation agent in PO-CL system.

In conclusion, HPLC-PO-CL determination method of H_2O_2 was developed for the first time and successfully applied to the assay of H_2O_2 in commercially available

ascorbic acid and cola drinks. Furthermore, the system was utilized for the determination of three commercially available peroxides (t-butyl hydroperoxide, cumene hydroperoxide, m-chloroperbenzoic acid) and H_2O_2 contaminated in them. The proposed H_2O_2 determination method is very sensitive and can detect as low as 188 fmol of H_2O_2. The method might be applied to assay of trace amounts of H_2O_2 generated in biological materials or environmental samples.

REFERENCES

1. G. Gubitz, P. Zoonen, C. Gooijer, N. H. Velthorst, R. W. Frei, Anal. Chem., 57: 2071-2074 (1985).

2. M. S. A.-Latif, G. G. Guilbault, Anal. Chem., 60: 2671-2674 (1988).

3. K. Nakashima, S. Kawaguchi, R. S. Givens, S. Akiyama, Anal. Sci., 6: 833-836 (1990).

4. K. Nakashima, N. Hayashida, S. Kawaguchi, S. Akiyama, Y. Tsukamoto, K. Imai, Anal. Sci., 7: 715-718 (1991).

5. M. Katayama, H. Takeuchi, H. Taniguchi, Anal. Lett., 24: 1005-1015 (1991).

6. K. Nakashima, S. Akiyama, Y. Tsukamoto, K. imai, Dyes Pigm., 12: 21-26 (1990).

7. K. Imai, Y. Matsunaga, Y. Tsukamoto, A. Nishitani, J. Chromatogr., 400: 169-176 (1987).

8. N. Imaizumi, K. Hayakawa, M. Miyazaki, K. Imai, Analyst, 114: 161-164 (1989).

9. N. Hanaoka, J. Chromatogr., 503: 155-165 (1990).

THE ANALYSIS OF IBUPROFEN ENANTIOMERS IN HUMAN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ON AN α1-ACID GLYCOPROTEIN CHIRAL STATIONARY PHASE

J. X. DE VRIES*, E. SCHMITZ-KUMMER, AND D. SIEMON

Medizinische Klinik der Universität Heidelberg
Abteilung für Klinische Pharmakologie
Bergheimer Strasse 58
D-69115 Heidelberg, Germany

ABSTRACT

A method for the quantitation of the enantiomers of the non-steroidal anti-inflammatory drug (NSAID) ibuprofen (IB) in human plasma and urine was developed for pharmacokinetic studies of the individual optical antipodes. Plasma samples were acidified, extracted with organic solvents and analysed by HPLC using an α_1-acid glycoprotein column and UV detection; elution was performed with a phosphate buffer and isopropanol gradient; RS-flurbiprofen (FL) was used as internal standard. Calibration curves were linear in the range 0.25 – 25 µg/ml of each IB-enantiomer. Enantiomers and internal standards were baseline separated. Precision and accuracy was ± 3-6%, the limit of detection 0.1 µg /ml, and the analytical recoveries of IB and FL 93.7 ± 5 % and 94.5 ± 4 % resp.; endogenous substances, IB metabolites and other drugs did not interfere with the assay. Urine samples were extracted and analysed as for plasma to assay free, and after alkaline hydrolysis, total IB-enantiomers.

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*Author to whom correspondence should be addressed.

The described assay is simple to perform, reproducible,
accurate and selective for the quantitation of IB-
enantiomers in plasma and urine without precolumn
derivatisation. Other chiral NSAID drugs were baseline
resolved under similar chromatographic conditions: FL,
fenoprofen and ketoprofen; the optical purities of
these compounds may be   determined with high
sensitivity with this HPLC system.

INTRODUCTION

The non-steroidal anti-inflammatory drug (NSAID)

ibuprofen   (α-Methyl-4-(2-methylpropyl)benzeneacetic

acid) (IB) is a widely used drug with analgesic, anti-

inflammatory and antipyretic properties; ibuprofen is

chiral but is marketed and administered to man as

racemic mixtures. In vivo the pharmacologic activity is

due mainly to the S-(+)-enantiomer and the R-(-)-isomer

is partially converted with inversion of the

configuration at the chiral center to S-(+)-IB; this

inversion has been proven to be unidirectional (1-3).

It has been suggested that drug stereoselective

disposition may be an important factor in the

individual therapeutic response to an NSAID (4,5).

Kinetics of ibuprofen is complex and cannot be defined

satisfactorily in terms of plasma total ibuprofen

concentration (6,7); therefore for a proper

understanding of stereochemical mechanisms of ibuprofen

disposition, it is necessary to measure the IB-

enantiomers.

Aim of the present work was to study the disposition of the ibuprofen enantiomers after analgesic dose administration of racemic ibuprofen in humans under different conditions and in patients with different disease states; for kinetic purpose an assay for the separate determination of the enantiomers in human plasma and urine was developed.

Several assays for the measurement of ibuprofen enantiomers in biological material have been recently described: direct analysis by HPLC with chiral stationary phases (8-10); HPLC after precolumn derivatisation with achiral (11) or chiral reagents (12-14); gas-chromatography - mass spectrometry of IB enantiomers after derivatisation with chiral amines (2,15).

In the present work plasma and urine samples were purified by solvent extraction and the ibuprofen enantiomers were analysed by HPLC using an α1-acid glycoprotein chiral stationary phase and UV detection; total amounts of ibuprofen enantiomers in urine were measured after alkaline hydrolysis; flurbiprofen was used as internal standard.

MATERIALS

Chemicals.

All chemicals were of analytical grade quality. Racemic ibuprofen, flurbiprofen, fenoprofen and

ketoprofen were available from Sigma (Deisenhofen,
FRG); pure (S)(+)- and R(-)-ibuprofen were gifts from
Prof. Dr. U. Klotz, (Dr. M. Fischer-Bosch Institut für
Klinische Pharmakologie, Stuttgart, FRG) and from
Sepracor Inc. (Marloborough, MA 01752, USA); N,N-
dimethyloctylamine (DMOA) (99%) was obtained from Fluka
Feinchemikalien (Neu-Ulm, FRG); 2-propanol was Uvasol
grade (Merck, Darmstadt, FRG). Solvents for HPLC were
filtered and degassed with helium before
chromatography.

Preparation of samples.

    a) Volunteers: A single oral 400 mg dose of
racemic ibuprofen (Aktren^R, (Bayer AG)) was
administered to 18 healthy subjects. For determination
of ibuprofen venous blood was collected in heparinized
tubes at time intervals till 12 h after administration;
the samples were cooled, immediately centrifuged and
the plasma was kept frozen at -20°C. Daily 24 h urines
were collected during three days and 20 ml aliquots
were kept frozen at -20°C. (b) Patients. A single 400
mg oral dose of ibuprofen was administeredTo twelve in-
patients with diagnostic liver cirrhosis and blood was
collected as described before.

    The studies were approved by the Ethics Committee
of the Faculty of Medicine, University of Heidelberg.

HPLC equipment.

For the assay of ibuprofen enantiomers a Model 1050 HPLC system consisting of a quaternary pumping system, autosampler, variable wavelength detector and a model 3396 Series II integrator (all from Hewlett Packard GmbH, Waldbronn, FRG) was used; the wavelength was programmed from 0 to 7 min at 220 nm, and from 7 to 15 min at 245 nm. For the determination of possible interferences with other drugs and peak purity tests a HP 1090 A System (Hewlett Packard) with photodiode array detection was used.

METHODS

Extraction procedure for plasma

1 ml plasma was mixed with 100 μl hydrochloric acid (1 M), 100 μl flurbiprofen (internal standard; 250 μg/ml), 100 μl water and 5 ml ether/hexane (2:8; v/v); the mixture was shaken for 10 min and centrifuged at 900 g for 5 min; 4 ml of the upper organic phase was transferred to a clean tube and evaporated under a stream of nitrogen at 20°C (water bath temperature). Before HPLC analysis, the extract was treated with 100 μl aqueous sodium hydroxide (0.01 M), sonicated for 3

min, followed by 50 μl phosphate buffer (0.1 M, pH 7.0,
with 0.1 % dimethyloctylamine) and sonication for 3
min; 5 μl were injected into the HPLC system.

Extraction procedure for urine

a) For the analysis of unconjugated ibuprofen the
samples were extracted as described for plasma; b) for
the analysis of total ibuprofen (conjugated and
unconjugated) 1 ml urine was treated with 500 μl sodium
hydroxide (1 M) for 30 min at room temperature; after
the addition of 700 μl hydrochloric acid (1 M), 100 μl
flurbiprofen (internal standard; 250 μg/ml) and 1 ml
water, the mixture was extracted with 5 ml ether/hexane
(2:8;v/v) and processed as described for plasma.

Enzymatic hydrolysis with β-glucuronidase/aryl
sulfatase were performed as described (16).

HPLC plasma and urine analysis.

5 μl plasma or urine extract solution was
injected into the HPLC system. Column: Chiral-AGP (100
x 4mm I.D., spherical 5 μm particle; ChromTech,
Norsborg, Sweden); guard column (10 x 3 mm) filled with
the same material. Eluent: solvent (A): 0.01 M aqueous
phosphate buffer pH 7.0 modified with 0.001 M

dimethyloctylamine; solvent (B): 2-propanol modified with 0.001 M dimethyloctylamine / 0.01 M aqueous phosphate buffer pH 7.0 modified with 0.001 M dimethyloctylamine (1:1; v/v). Solvent flow rate: 0.9 ml / min. Gradient elution system 1: 0-5 min isochratic with 0.8% B; 5-15 min linear gradient from 0.8 to 41% B; reequilibration time 10 min; the operating pressure and temperature were 10-12 MPa and 20-25°C respectively. After an analysis series the column was washed with an aqueous 10% 2-propanol solution during 15 min. Peak areas of (R) and (S) ibuprofen, and peak 2 of the internal standard (Figures 1 and 2) were integrated and the concentrations were calculated using the corresponding calibration factors.

Preparation of standards.

Plasma: Standard stock solutions of (RS) ibuprofen (10.00 mg in 10 ml 0.01 M sodium hydroxide) and (RS) flurbiprofen (2.50 mg in 10 ml 0.01 M sodium hydroxide) were prepared and maintained one week in the dark at 2-8°C. The ibuprofen stock solution was diluted with sodium hydroxide (0.01M) to concentrations of 5 to 500 μg / ml. 100 μl of the diluted solutions were added to 1 ml pretreatment plasma to obtain final ibuprofen plasma concentrations of 0, 0.25, 0.5, 1.25, 2.5, 5,
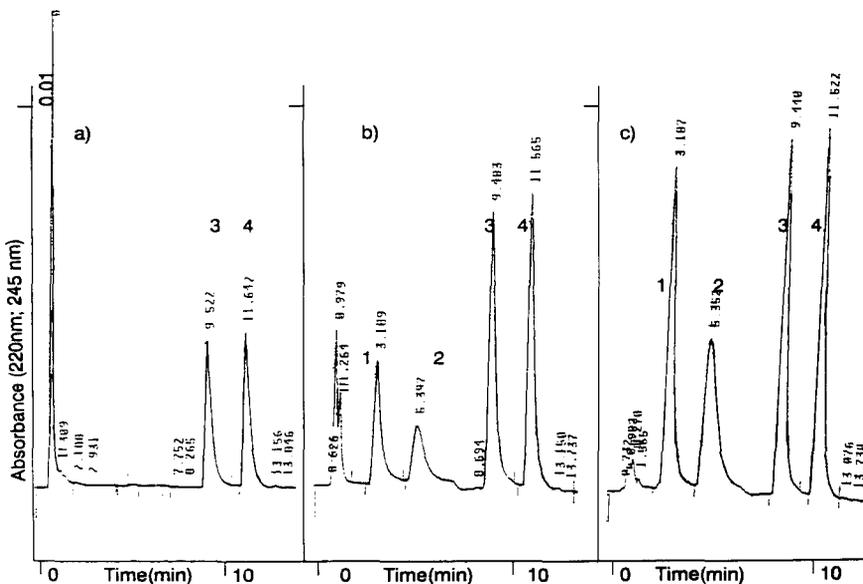
FIGURE 1. HPLC analysis of a plasma extract: a) control
plasma; b) control plasma after addition of 10 μg /ml
of (R) and (S) ibuprofen   c) plasma sample from a
volunteer 45 min after a 400 mg single oral dose of
ibuprofen (concentrations of 15.1 and 16.0 μg /ml of
(R)-and (S) -ibuprofen resp.). 25 μg / ml of racemic
flubiprofen were added to all samples; 1 and 2
correspond to (R)- and (S)-ibuprofen enantiomers; 3 and
4 to the flurbiprofen enantiomers.

12.5 and 25 μg / ml of each enantiomer; after the

addition of 100 μl flurbiprofen (internal standard;

250 μg/ml) and 100 μl water, the samples were extracted

as described under plasma extraction procedure. *Urine*:

The same ibuprofen and flurbiprofen stock solutions and

dilutions as described for plasma were used . To 1 ml
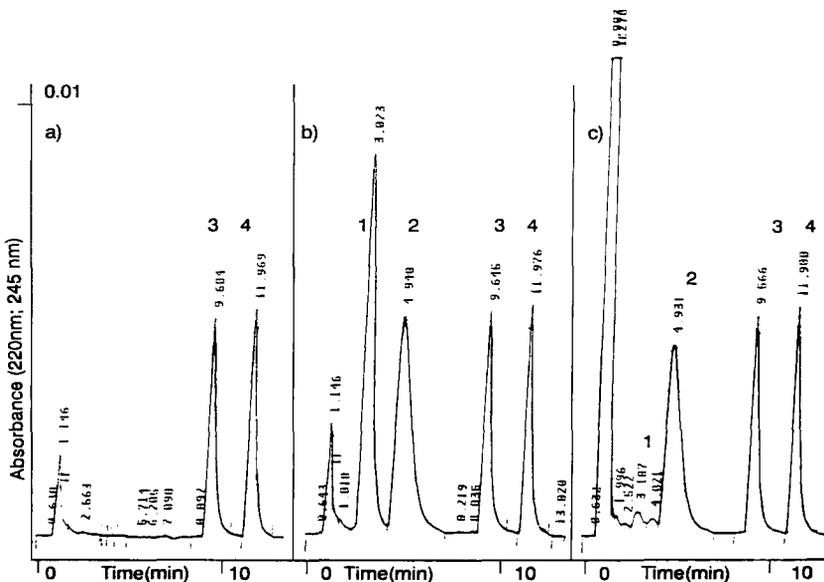
FIGURE 2. HPLC analysis of an urine extract after alkaline hydrolysis: a) control urine; b) control urine after addition of 100 µg / ml of (R)-and (S)-ibuprofen c) 0-24 h urine sample from a volunteer after a 400 mg single oral dose of ibuprofen (concentrations of 2.0 and 40.2 µg /ml of (R)- and (S)-ibuprofen resp.); 25 µg / ml of racemic flubiprofen were added to all samples. 1 and 2 correspond to (R)- and (S)-ibuprofen enantiomers; 3 and 4 to the flurbiprofen enantiomers.

urine, the corresponding amounts of ibuprofen and internal standard were added as for plasma in the ibuprofen concentration range 0 - 100 µg / ml, and processed as described under extraction procedure for urine a) or b).

Calibration curves.

Calibration curves for plasma and urine analysis were obtained by processing plasma and urine with known amounts of (R) and (S) ibuprofen and flurbiprofen as mentioned above, before every analysis series. Least-squares linear regression of the ratio of: ((R) (or (S)) peak area) / (flurbiprofen peak 2 area) vs. added (R) (or (S)) ibuprofen concentrations were used to calculate the calibration factors.

HPLC separation of NSAIDs enantiomers

1 μl of solutions of ibuprofen, flurbiprofen, fenoprofen and ketoprofen (1 mg / ml in methanol) were analysed by the HPLC equipment and column described above; Gradient elution system 2: linear gradient from 0 to 40% B in 10 mion; reequilibration time 10 min.

<u>RESULTS</u>

Endogenous substances, ibuprofen metabolites, numerous drugs and their metabolites were tested for possible chromatographic interferences (see (16)) and were found not to interfere with the assay; the first peak of ketoprofen showed similar retention times as

TABLE 1

Column capacity factors (k') and separation factors (α) for the
enantiomeric separation of ibuprofen and other NSAIDs. See methods
for the description of chromatographic parameters.

Compound	Elution system 1			Elution system 2		
	k'_1	k'_2	α	k'_1	k'_2	α
(RS)-Ibuprofen	1.77	3.24	1.83	1.70	2.08	1.22
(S)-Ibuprofen	---	3.27	---	---	2.16	---
(R)-Ibuprofen	1.75	---	---	1.73	---	---
(RS)-Flurbiprofen	8.26	10.46	1.27	3.36	5.21	1.55
(RS)-Fenoprofen	5.08	7.75	1.52	2.53	2.95	1.16
(RS)-Ketoprofen	3.49	6.38	1.82	2.18	2.57	1.18

(S)-ibuprofen in both elution systems 1 and 2 (Table
1).

Fig.1 shows chromatograms from plasma extracts:
1a) from pretreatment plasma, 1b) from pretreatment
plasma after the addition of 10 μg/ml of each (R) and
(S) ibuprofen, and 1c) from a subject 45 min after
administration of an oral dose of 400 mg ibuprofen
racemate. Enantiomers were baseline separated: peak
assignments for the (R) and (S) antipodes of ibuprofen
were obtained by comparing the HPLC retention times
after the injection of the pure enantiomers with known
absolute configuration (Table 1); pure flurbiprofen
enantiomers of known configuration were not obtainable;
retention times were constant (± 2%) during an analysis
series . A within-run precision of ± 3-6% relative
standard deviation was found; the calculated analytical
recoveries were 94 % for ibuprofen and 96 % for

flurbiprofen at a concentration of 5 and 12.5 μg / ml of each enantiomer, respectively. Calibration curves were linear in the range 0.25 - 25 μg / ml for each enantiomer. The limit of detection, defined as 3σ above the average blank was 0.1 μg /ml; no racemisation during the procedure could be detected by analysing pretreatment plasma samples after addition of pure (R) or (S) enantiomers.

Chromatograms from urine samples after alkaline hydrolysis are shown in Fig. 2; Fig 2a of pretreatment sample, 2b) from a pretreatment urine after the addition of 100 μg / ml of each ibuprofen enantiomer,and 2c) shows the chromatogram of a urine sample collected in the first 24 h after ibuprofen administration . The within-run precision, the calculated analytical recoveries, selectivity were similar to plasma. The limit of detection was 0.25 μg / ml and the calibration curves were linear in the range 0.25 - 25 μg / ml for each enantiomer . Total ibuprofen (conjugated and unconjugated) was obtained hydrolysing urine with alkali; this procedure showed higher analytical recoveries of free (R) and (S) ibuprofen as the enzymatic hydrolysis with ß-glucuronidase / aryl sulfatase and did not show any racemization with samples containing pure (R) or (S) ibuprofen. The mean cumulative urine elimination of

total (conjugated and unconjugated) (R) and (S) ibuprofen in healthy subjects was shown to be 1.17 and 19.0% of the original ibuprofen (R) and (S) dose; unconjugated ibuprofen enantiomers were eliminated only in trace amounts (< 0.5% of the dose).

The peak purities of IB and FL in plasma and urine extracts from subjects after IB administration, were determined by HPLC using a photodiode array detector; the UV spectra of the ascending, apex and descending parts of the curve were superposable with those of the pure compounds.

The enantiomers of racemic fenoprofen and ketoprofen were also resolved by HPLC (Table 1).

DISCUSSION

The need of analysing large numbers of plasma and urine samples for pharmacokinetic studies on the disposition of ibuprofen enantiomers after racemate drug administration, lead us to reevaluate HPLC assays reported in the literature (8,9,10,11). The method described in the present work used direct HPLC analysis with an α_1-acid glycoprotein column after solvent extraction and showed advantages with respect to the reported ones.

No precolumn derivatisation of ibuprofen with chiral or achiral reagents was necessary; own attempts

to derivatise IB as described in (11) were lengthy and showed interfering artifact peaks originating from side reactions with endogenous substances.

Racemic flubiprofen, of similar chemical structure as IB was used as internal standard for better precision and accuracy; no pure FL enantiomers of known configuration were available for peak assignment. The use of $\alpha 1$-acid glycoprotein as a chiral stationary phase has been reported for the enantiomeric separation of a large number of drugs (17-19, including ibuprofen (9,10). In the present work, IB and the internal standard FL were resolved with base line separation of the enantiomers with good reproducibility and stability; these peaks did not interfere with endogenous substances or other drugs; with careful column handling the column was still useful after more than 800 injections of biological extracts. Experiments with a ß-cyclodextrine phase (8) did not lead to IB enantiomer baseline separation.

The ibuprofen peaks were detected at the absorption maximum 220 nm, and with UV detector time-wavelength programming the internal standard peaks were detected at their absorption maximum at 245 nm; the UV detection was combined with isochratic elution of the ibuprofen peaks, and a gradient elution of the flurbiprofen peaks which allowed a good column selectivity, adequate sensitvity and running times.

The liquid extraction procedure was simple to perform and showed good analytical recoveries and no chromatographic interferences; low boiling organic solvents were used and the evaporation step was performed at low temperatures as ibuprofen is quite volatile, a fact that is nowhere mentioned; IB extracts must be injected into the HPLC system in aqueous solutions, as band broadening of chromatographic peaks occur when the sample is injected in organic solvents (methanol, isopropanol). Attempts to purify plasma with solid phase extractions of ibuprofen with C_{18} phases showed erratic and non reproducible results.

Potential racemization of IB during the whole analytical procedure was controlled analysing samples containing pure IB enantiomers; no detectable racemization occurred. Pharmacokinetic parameters for plasma were similar to those reported in the literature (11).

The method was also used for the analysis of urine samples; free ibuprofen enantiomers were analysed by direct extraction without hydrolysis; total ibuprofen enantiomers were analysed after alkaline hydrolysis; alkaline hydrolysis showed higher analytical recoveries as the enzymatic hydrolysis with ß-glucuronidase / arylsulfatase and no racemization was detectable; as IB is extensively metabolised by the

liver (1,2,3) only a fraction of the drug is recoverd in urine , mainly as conjugates (20).

The enantiomers of other chiral NSAID drugs (flurbiprofen, fenopfrofen and ketoprofen) were baseline resolved under similar chromatographic conditions (Table 1): However, the pure enantiomers with known absolute configuration were not available and no peak could be assigned; the optical purities of these compounds may be determined with high sensitivity with this HPLC system.

(R) and (S) ibuprofen plasma concentrations were measured in patients with liver cirrhosis and in healthy subjects; calculation of pharmacokinetic parameters showed higher average areas under the plasma concentration-time curves ($AUC_{0-\infty}$) (+37% (R), + 27% (S)) and longer plasma elimination half-lives (+40% (R), +44% (S)) for patients with cirrhosis when compared with data from healthy individuals. This indicates an impairment in the elimination of both enantiomers in cirrhosis patients. The mean ratio of ($AUC_{0-\infty}$)(S)/($AUC_{0-\infty}$)(R) was lower in patients than in healthy subjects, indicating an impairment in the inversion of (R) into (S) in patients (21). Similar results were reported by Li et al. (22), who used another assay for the determination of ibuprofen enantiomers.

In summary, a simple, reproducible, accurate and selective method was developed for the analysis of ibuprofen enantiomers in plasma and urine extracts with a direct chiral stationary phase and UV detection was developed for pharmacokinetic studies in humans. The enantiomers of other NSAID may also be separated, and the optical purities of the antipodes determined with high accuracy and sensitvity.

ACKNOWLEDGMENTS

REFERENCES

1. H.B. Hucker, K.C. Kwan, D.E. Duggan, "PHARMACOKINETICS AND METABOLISM OF NON-STEROIDAL ANTI-INFLAMMATORY AGENTS" in "Progress in Drug Metabolism, J.W. Bridges, L.F. Chasseaud, (eds.), J. Wiley & Sons, Chichester, U.K., 1980, Vol. 5, pp. 165-253

2. T.A. Baillie, W.J. Adams, D.G. Kaiser, L.S. Olanoff, G.W. Halstead, H. Harpootlian, G.J. Van Giessen, J. Pharmacol. Exp. Ther., 249: 517-523 (1989)

3. R.O. Day, G.G. Graham, K.M. Williams, G.D. Champion, J. De Jager, P.M. Brooks, Pharmacol. Ther. 33: 383-433(1987)

4. R.O. Day, P.M. Brooks, Br. J. Clin. Pharmacol., 23: 655-658 (1987)

5. K.M. Williams, R.O. Day, Agents Actions, 17 (Suppl): 119-126 (1985).

6. E.J.D. Lee, K. Williams, R. Day, G. Graham, D. Champion, Br. J. Clin. Pharmacol., 19: 669-674 (1985)

7. A. Avgerinos, A.J. Hutt, Chirality, 2: 249-256 (1990)

8. G. Geisslinger, K. Dietzel, D. Loew, O. Schuster, G. Rau, G. Lachmann, K.Brune, J. Chromatogr., 491: 139-149 (1989).

9. S. Menzel-Soglowek, G. Geisslinger, K. Brune K, J. Chromatogr., 532: 295-303 (1990)

10. K.J. Pettersson, A. Olsson, J. Chromatogr., 563: 414-418 (1991)

11. G. Li, G. Treiber, U. Klotz, Drug Investigation, 1: 11-17 (1989)

12. A.J. Hutt, S. Fournel, J. Caldwell, J. Chromatogr., 378: 409-418.(1986)

13. E.J.D. Lee, K. Williams, R. Day, G. Graham, D. Champion, J. Pharm. Sci., 73: 1542-1544 (1984).

14. A. Avgerinos, A.J. Hutt, J. Chromatogr., 415: 75-83 (1987)

15. D.G. Kaiser, G.J. Van Giessen, R.J. Reischer, W.J. Wechter, J. Pharm. Sci., 65: 269-273 (1976).

16. J.X. de Vries, J. Harenberg, E. Walter, R. Zimmermann, M. Simon M, J. Chromatogr., 231: 83-92 (1982).

17. M. Enquist, J. Hermansson, J. Chromatogr., 519: 285-298 (1990).

18. J. Hermansson, M. Enquist, J. Liquid Chromatogr., 9: 621-639 (1986).

19. J. Hermansson, J. Chromatogr., 269: 71-80 (1983).

20. M. Spraul, M. Hofmann, P. Dvortsak, J.K. Nicholson, I.D. Wilson, Anal. Chem., 65: 327-330 (1993)

21. V. Hofmann, J. de Vries, R. Raedsch, D. Siemon, I. Walter-Sack, Klin. Pharmakol. akt., 4: 25 (1993).

22. G. Li, G. Treiber, K. Maier, S. Walker, U. Klotz, Clin. Pharmacokinet., 25: 154-163 (1993).

HPLC DETERMINATION OF VERAPAMIL AND NORVERAPAMIL IN PLASMA USING AUTOMATED SOLID PHASE EXTRACTION FOR SAMPLE PREPARATION AND FLUOROMETRIC DETECTION

Ph. HUBERT AND J. CROMMEN
Laboratory of Drug Analysis
Institute of Pharmacy
University of Liège
rue Fusch, 5
B-4000 Liège, Belgium

ABSTRACT

A sensitive and fully automated method for the simultaneous determination of verapamil and its main metabolite norverapamil in plasma is described, which involves the solid phase extraction (SPE) of the analytes from plasma on disposable extraction cartridges (DECs) and reversed phase HPLC with fluorescence detection. The DEC filled with endcapped cyanopropyl silica (50 mg) was first conditioned with methanol and phosphate buffer of pH 7.4. A 1.0-mL volume of plasma sample containing the internal standard was then applied on the DEC. The washing step was performed with the same buffer. The analytes were eluted with 0.24 mL of methanol containing 0.2 % of 2-aminoheptane. A 0.41-mL volume of acetate buffer of pH 3.0 was then passed through the DEC and 0.25 mL of the resultant extract was directly introduced into the HPLC system. The absolute recoveries of the drugs were around 95 % and the limit of detection of verapamil was 1.0 ng/mL. The within-day and between-day reproducibilities at a plasma concentration of 100 ng/mL were 1.4 % and 1.9 %, respectively.

INTRODUCTION

Verapamil hydrochloride (cfr. Fig. 1), a synthetic papaverine derivative, is a well known calcium blocker compound which has anti-anginal, anti-hypertensive and anti-arrhythmic properties [1]. This drug is extensively metabolized. Norverapamil, its N-demethylated metabolite (cfr. Fig. 1), is pharmacologically active and can accumulate in plasma concentrations equal to or greater than those of verapamil itself [2].

Verapamil and its metabolites have been analyzed previously by conventional gas chromatography with flame ionization detection [3,4] or mass spectrometry [5], more recently by capillary gas chromatography with flame ionization detection or electron capture detection [6] and by high performance liquid chromatography using UV detection [6] or most often fluorescence detection [2,7-12] owing to the native fluorescence properties of these compounds.

A sample handling step is usually introduced prior to the HPLC analysis of drugs in biological fluids in order to remove proteins which may cause column clogging [13,14] and also to increase the selectivity and sensitivity of the method [15,16]. In most analytical procedures mentioned above, the sample pretreatment consists of liquid-liquid extraction of the analytes from plasma by organic solvents [10] after alkalization [2,6]. This is generally followed by a back-extraction into acidic solutions [3,4,7,8,9,11,12]. An alternative to this efficient but often tedious and time-consuming extraction technique is the isolation of drugs by liquid-solid or solid-phase extraction (SPE) using either column switching [13,14,16-20] or disposable extraction cartridges (DECs) [15,20-23]. The different SPE operations can be performed automatically on DECs by means of a sample processor, which can also inject the prepared sample on line into the HPLC system [23,24].

The purpose of this paper is to describe such a fully automated method developed for the determination of verapamil and its main metabolite norverapamil in plasma. The method involves sample handling by SPE on DECs, on-line injection of the extracts into a HPLC system and subsequent fluorometric detection of the analytes. The influence of the competing amine added to the mobile phase on the background fluorescence has been studied and the differents steps of the SPE procedure which may effect analyte recovery

	R_1	R_2
(1)	CH_3	H
(2)	H	H
(3)	CH_3	OCH_3
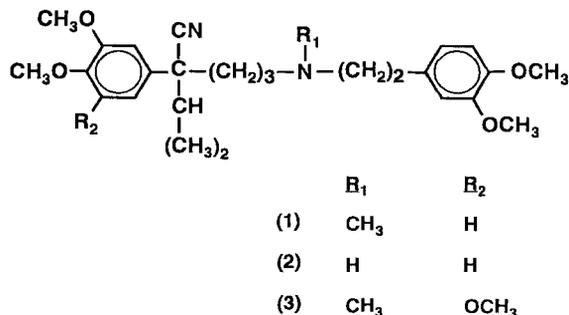
FIGURE 1

Structure :
(1) : Verapamil; (2) : Norverapamil; (3) : Gallopamil (internal standard)

have been investigated using aqueous standard solutions instead of spiked plasma samples in order to limit the consumption of DECs [25].   After elimination of memory effects and selection of the most appropriate dispensing mode for the liquids in the elution step, the composition of the eluent and of the washing liquid, the type of SPE sorbent and the volume of eluent have been optimized with respect to analyte recovery. Finally, the method developed has been validated.

MATERIALS AND METHODS

Apparatus

The chromatographic system consisted of a model 305 pump (Gilson, Villiers-le-Bel, France), an ASPEC system (Automatic Sample Preparation with Extraction Cartridges) from Gilson and a model F-1050 fluorescence detector from Merck-Hitachi (Darmstad, FRG / Tokyo, Japan) equipped with a mercury-xenon lamp (200 W). The fluorometer was set at an excitation wavelength of 275 nm, an emission wavelength of 310 nm [4,12] and a time constant of 1 sec.

The ASPEC system was composed of three components: an automatic sampling injector module, a model 401 dilutor/pipettor and a set of racks and accessories, necessary for handling DECs, plasma samples and solvents [23,24].

A Manu-CART system which was made up of a LiChroCART analytical column (250 x 4 mm, i.d.) preceded by a short LiChroCART guard column (4 x 4 mm, i.d.) from Merck (Darmstad, FRG), was thermostatted at 35.0 $\pm$ 0.1 ° C in a model 02PT923 water-bath from Heto (Birkeröd, Denmark).

An IBM compatible computer (PC-AT; CPU type 80386) equipped with GME-714 version 1.3 (HPLC system controller) and GME-718 version 1.1 (sample manager) softwares from Gilson, was used to control the HPLC and ASPEC systems as well as for data collection, storage and treatment. A model BD9 two-channel recorder from Kipp and Zonen (Delft, The Netherlands) was used simultaneously for data collection.

Chemicals and Reagents

R,S-verapamil hydrochloride, its N-demethyl derivative (norverapamil) and gallopamil (cfr. Fig. 1), the internal standard, were all kindly supplied by S.M.B Pharmaceuticals (Brussels, Belgium) and were used without further purification.

Sodium acetate, glacial acetic acid, sodium hydroxide and potassium dihydrogen phosphate were of p.a. quality from Merck (Darmstad, FRG). 2-Aminoheptane was purchased from Aldrich (Gillingham, Great Britain) and was distilled two times before use.

Acetonitrile from Riedel-de Haen (Seelze, FRG) and methanol from Janssen (Geel, Belgium) were of HPLC grade.

The water used in all experiments was purified on a Milli-Q system from Millipore (Bedford, MA, USA).

Bond Elut DECs (1-mL capacity) packed with 50 mg of cyanopropyl modified silica (CN endcapped: CN^{EC}) with a mean particule size of 40 μm were obtained from Analytichem (Harbor City, CA, USA).

The stationary phase of the LiChroCART analytical column was Superspher 100 RP-18 (particule size : 4 μm) and the LiChroCART guard column was packed with LiChrospher 100 RP-18 (particule size : 5 μm) from Merck.

Chromatographic technique

The mobile phase (isocratic mode) was a mixture of pH 3.0 acetate buffer, acetonitrile and 2-aminoheptane (70:30:0.5, v/v/v) [26]. Before use, it was degassed for 15 min. in a ultrasonic bath. The flow-rate of the mobile phase was 1.1 mL/min.

The acetate buffer of pH 3.0 was obtained by mixing 0.01 N sodium acetate with 33 mL of glacial acetic acid per liter, the pH being adjusted if necessary. The buffer was filtered through a 0.45 μm nylon membrane filter from Schleicher & Schuell (Dassel, FRG) before use.

Standard solutions

Stock solutions of verapamil, norverapamil and gallopamil (internal standard) were prepared by dissolving 50 mg of each compound in 50 mL of methanol. A mixed verapamil/norverapamil solution (10 μg/mL for each compound) was made in water and diluted either with water (concentrations ranging from 0.03 to 3.00 μg/mL) when used to spike plasma samples (1.5 mL) for calibration curves or with a mixture of pH 3.0 buffer and methanol (70:30) when injected directly for recovery measurements. The stock solution of gallopamil was diluted with water to the concentration of 5 μg/mL.

The methanolic solutions were stored in a refrigerator at 4 °C while the aqueous solutions were prepared daily.

Automatic sample preparation

After centrifugation of the plasma sample at 6000 rpm for 10 min., a 1.5-mL volume of plasma was transferred manually into a vial which was then placed on the appropriate rack of the ASPEC system. All the other operations of the SPE procedure and the injection of the final extract into the HPLC system were performed automatically by the sample processor (ASPEC system). As outlined in Table 1, the automatic operations are performed in the following way (total cycle time : 20.4 min) :

- **Addition of internal standard solution** (flow rate : 3.0 mL/min; air volume : 1.0 mL) : after addition of 0.03 mL-volume of gallopamil solution, the plasma sample is homogenized by bubbling.
- **DEC conditioning** (flow rate : 6.0 mL/min; air volume : 0.3mL) : at the beginning of the cycle, the DEC holder is located above the drain

TABLE 1

Automatic Sample Preparation Procedure

Steps	Liquids	Volume (mL)	Dispensing flow rate (mL/min)
IS. Addition	IS Solution	0.03	3.00
Conditioning	Methanol	1.00	6.00
	B. pH 7.4	1.00	6.00
Sample loading	Plasma	1.00	0.18
Washing	B. pH 7.4	1.00	1.50
Elution	CH3OH+0.2% AH	0.24	1.50
Buffer addition	B. pH 3.0	0.41	1.50
Mixing	Plasma extract	0.65	3.00
Filling of the injection loop	Plasma extract	0.65	0.75

DEC : Bond Elut CN^{EC} (50 mg; 1 mL capacity)
B. : Buffer   IS: Internal Standard   AH: 2-Aminoheptane

cuvette (front position). The DEC (Bondelut CN^{EC}, 50 mg) is first washed with 1.0 mL of methanol , then with 1.0 mL of phosphate buffer (pH 7.4).

- **Loading with plasma sample** (flow rate : 0.18 mL/min; air volume : 1.0 mL) : 1.0 mL-volume of plasma is aspirated by the autosampler needle from the corresponding vial and dispensed on the DEC.

- **Washing** (flow rate : 1.5 mL/min; air volume : 1.0 mL) : 1.0 mL of phosphate buffer pH 7.4 is dispensed on the DEC.

- **Elution** (flow rate : 1.5 mL/min; air volume : 1.0 mL) : the DEC holder is pushed by the needle over the collection rack. A 0.24-mL volume of methanol containing 0.2 % of 2-aminoheptane is applied on the DEC. The eluate is collected in the tube located under the DEC.

- **Dilution** (flow rate : 1.5 mL/min; air volume : 1.0 mL) : 0.41 mL of acetate buffer (pH 3.0) are dispensed on the DEC. The DEC holder is then replaced in its front position.

- **Mixing** : the diluted eluate is successively aspirated and dispensed in the collection tube by the needle. These operations are repeated three times.

- **Injection** : the whole volume of the final extract (0.65 mL) is aspirated by the needle from the collection tube and dispensed in the loop filler port of the injection valve. By switching of the injection valve, 0.25 mL of the final extract is injected into the HPLC column, the excess being directed to the waste.

In order to avoid detrimental effects of the strong binding of the analytes to plasma proteins on the absolute recoveries, the minimum dispensing flow rate available (0.18 mL/min) has been selected for the sample loading step [23].

The phosphate buffer of pH 7.4 which was used in the conditioning and washing steps, was prepared in a 1-L volumetric flask by mixing 250 mL of 0.1M potassium dihydrogen phosphate with 195.5 mL of 0.1 M sodium hydroxide and adding water to the volume.

Each plasma sample was prepared individually during the chromatographic analysis of the previous sample (concurrent mode).

Before the beginning of the first cycle and after each use in the SPE procedure, the needle of the autosampler was rinsed with water and a 40-μL segment of air was generated before pipetting the liquid to be transferred in order to avoid cross-contamination.

RESULTS AND DISCUSSION

Fluorometric detection

In the development of a bioanalytical procedure, a proper choice of the detection mode is particulary important. Due to their higher sensitivity and selectivity, fluorescence and electrochemical detection are usually preferred to UV-VIS detection, especially when no derivatization step is needed. Verapamil and its main metabolite, norverapamil, as well as gallopamil (I.S.) have native fluorescence properties, which makes fluorometry an obvious choice in this case. The main interest of this detection mode in the present method is the

substantial gain of sensitivity which can be obtained in comparison with UV detection, a high degree of selectivity being already provided by the SPE procedure and the HPLC separation [27].

The sensitivity of fluorescence detection could be further improved if the conventional xenon lamp (150 W) of the detector was replaced by a mercury-xenon lamp (200 W), particularly at excitation wavelengths lower than 300 nm. For the compounds studied, the fluorescence signal at 310 nm (excitation wavelength : 275 nm) was then seven times higher [28].

As shown in Fig. 2, 2-aminoheptane, added to the HPLC mobile phase to improve analyte peak symmetry and efficiency, gave rise to a significant increase of the background fluorescence. When the amine was used as supplied by the manufacturer, a constant baseline was obtained only after passage of more than 400 mL of mobile phase. By monitoring the HPLC eluate (cfr. Fig. 2), several breakthrough curves were observed, indicating the presence of at least five fluorescent impurities with widely different hydrophobic character. The equilibrium was reached more rapidly by use of the sulphate salt of the amine but the background fluorescence was only slightly diminished. The latter could be considerably reduced, however, by distillation of the amine, which eliminated mainly the most retained impurities and consequently also decreased the equilibrium time.

Memory effects

Table 2 shows the detrimental influence of memory effects on repeatability by direct injection of aqueous standard solutions of verapamil and norverapamil into the HPLC system. The use of a pH 7.4 phosphate buffer for the preparation of standard solutions of the analytes as well as for rinsing the dilutor needle and the external tubing of the injection valve gave rise to very high RSD values of about 10 % (n=5). Under these conditions, the total amount of the analytes was not introduced into the HPLC system, as it was demonstrated by injecting successively five samples of a aqueous standard solution and then a mixture of pH 3.0 acetate buffer and methanol (70:30, v/v) containing no analytes. On the chromatogram corresponding to the injection of this mixture, small residual peaks were observed at the retention times of the two analytes. The replacement of the 7.4 buffer by a pH 3.0 acetate buffer as rinsing liquid did not improve significantly the RSD values (cfr. Table 2).
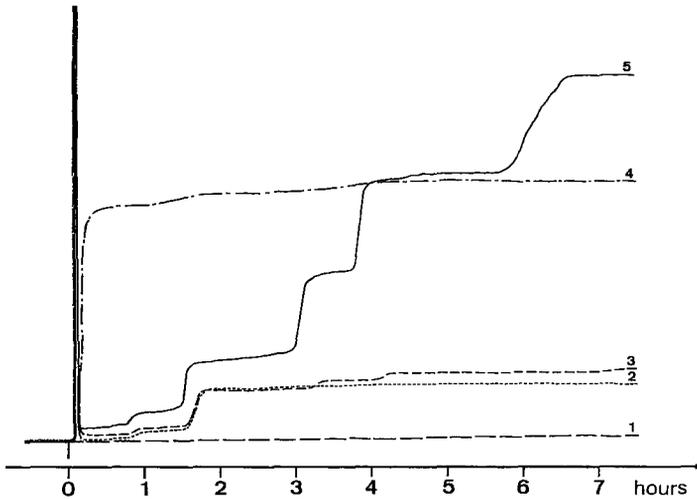
FIGURE 2

Fluorescence monitoring of the HPLC eluate
0.5 % 2-Aminoheptane in phosphate buffer pH 3-Acetonitrile (70/30)
 - (1) : mobile phase without the amine
 - (2) : amine distilled twice
 - (3) : amine distilled once
 - (4) : sulphate salt of the amine
 - (5) : unpurified amine

As can be seen in Table 2, the best results with respect to repeatability were obtained with standard solutions of the analytes prepared in a mixture of pH 3.0 acetate buffer and methanol (70:30, v/v). In spite of the particularly good results obtained with pH 3.0 buffer as rinsing liquid, water was finally given the preference due to the unfavourable effect of this buffer on the recovery of verapamil and norverapamil (cfr. Table 4).

It should be noted that plasma samples spiked with the same amounts of analytes and treated successively according to the SPE procedure described in Materials and Methods gave reproducible results (cfr. Table 7) by use of water

TABLE 2

Memory Effects Obtained by Direct Injection

| Aqueous standard solution of drugs	Dilutor/ pipettor rinsing liquid	R.S.D. (%; n=5)		Residual peak
		NV	V	
B. pH 7.4	B. pH 7.4	10.3	9.2	+
B. pH 7.4	B. pH 3.0	4.1	6.5	+
B. pH 3.0 /CH3OH	B. pH 3.0	0.8	0.4	-
B. pH 3.0 /CH3OH	Water	1.4	1.5	-

NV : Norverapamil   V : Verapamil   B. : Buffer
B. pH 3.0/CH3OH : Acetate buffer pH 3.0 - Methanol (70:30)
Analyte concentration : 100 ng/mL

as rinsing liquid and that no residual peaks were observed when a blank plasma sample was analyzed immediately afterwards under the same conditions.

Dispensing mode for the liquids in the elution step

In the development of the SPE procedure by use of aqueous standard solutions of the analytes, the elution of these compounds from the extraction cartridges was the first step to be studied. At this stage, the different SPE steps had not yet been optimized. As selected previously in a similar fully automated method for the bioanalysis of another basic drug, diltiazem [24,27], DECs filled with ordinary cyanopropyl silica were used, as well as a pH 7.4 phosphate buffer for the conditioning and washing steps. The elution was first performed with a 0.3-mL volume of methanol (cfr. Fig. 3) and 0.7 mL of acetate buffer
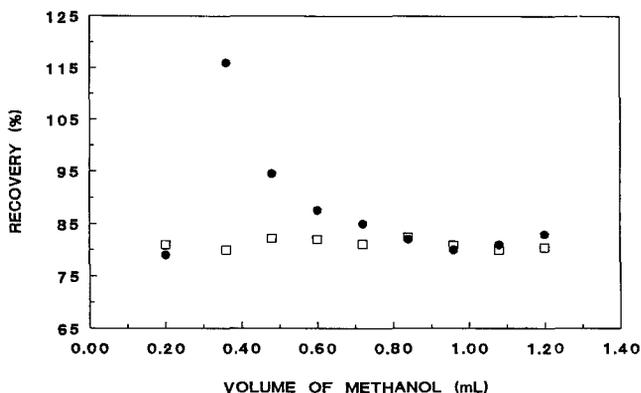
FIGURE 3

Influence of the dispensing mode of phosphate buffer (pH 7.4) on the recovery
of verapamil

DEC : Bond Elut cyanopropyl-bonded phase (CN; 50 mg)
Verapamil concentration : 100 ng/mL
Conditioning liquids : 1. Methanol 2. Phosphate buffer pH 7.4
Washing liquid : Phosphate buffer pH 7.4
Buffer added to the methanolic eluate : pH 3.0 acetate buffer
(●) : dispensed in the collection tube
(□) : dispensed on the DEC

(pH 3.0) was subsequently delivered into the corresponding collection tube
located under the extraction cartridge. The aim of this buffer addition is to
reduce the eluting strength of the final extract to such an extent that it becomes
equivalent or preferably lower in comparison with that of the HPLC mobile
phase [27].

Fig. 3 shows that under these preliminary conditions anomalously high
(more than 110%) and poorly reproducible recoveries were obtained for
verapamil at the 100 ng/mL concentration level. On the other hand, the use of
methanol volumes higher than 0.7 mL gave rise to lower (around 80 %) but
more reproducible recoveries.

It can also be seen in Fig. 3 that when acetate buffer was passed through the DEC just after the application of methanol, this unfavourable effect was eliminated and a fairly constant recovery of about 80 % was obtained for all methanol volumes tested. In all these experiments, the amount of buffer added always corresponded to 70 % of the final extract, i.e. the same proportion as in the HPLC mobile phase.

These results can be explained by the fact that if only methanol is applied on the DEC and acetate buffer is introduced directly in the collection tube, the volume of eluate collected is lower than the methanol volume dispensed. This is particularly obvious when the latter is small. The passage of the buffer through the DEC is thus necessary, not to improve the elution of the analytes, but to obtain a constant volume of eluate, equivalent to the volume of methanol dispensed. Even small volumes of methanol could be restored under these conditions (cfr. Fig. 4), so that this dispensing mode was selected for further experiments.

Composition of the eluent

As can be seen in Table 3, solvents often used in reversed-phase chromatography such as acetonitrile and methanol were first tested as eluents. With acetonitrile, particularly low recoveries of about 20 % were obtained for verapamil and norverapamil. Methanol gave, however, significantly higher recoveries than acetonitrile. In contrast to what is usually observed in reversed-phase HPLC, acetonitrile seems to have a lower eluting strength than methanol under these conditions. Similar observations have been reported previously by solid phase extraction of basic compounds [29-31]. The results obtained here with acetonitrile are probably to be related to the strong tendency of basic compounds to interact with free silanol groups, particularly when the solid phase consists of ordinary cyanopropyl silica.

Still the recoveries of about 80 % obtained with methanol as eluting solvent are not quite satisfactory. The addition to methanol of 2-aminoheptane, i.e. the same competing amine as used in the HPLC mobile phase, gave rise to a significant increase in the recoveries of the two analytes, at a concentration to 0.2 % (cfr. Table 3). No further increase in recovery were obtained, however, by adding higher amounts of 2-aminoheptane to methanol (e.g. 0.4 %). The favourable influence of the competing amine on the elution of verapamil and
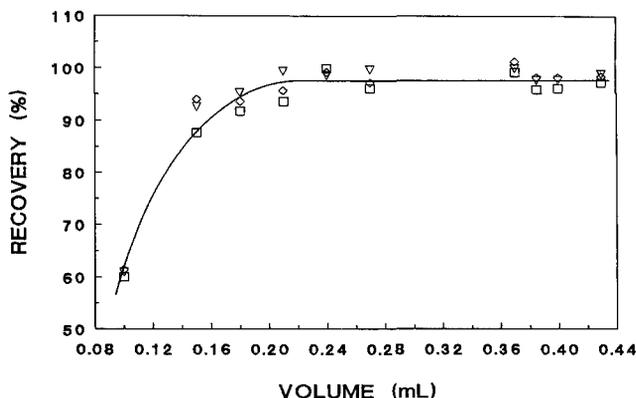
FIGURE 4

<u>Minimum volume of eluent for the elution step</u>

DEC : Bond Elut CN endcapped (CN^{EC}; 50 mg)
Eluent : methanol containing 0.2 % of 2-aminoheptane
Analytes concentration : 100 ng/mL
(∇) : norverapamil
($\diamond$) : verapamil
($\square$) : gallopamil
Other conditions as described in Materials and Methods.

norverapamil seems to confirm that these compounds have strong interactions with the residual silanol groups at the surface of the solid phase.

<u>Composition of the washing liquid</u>
        Table 4 shows the influence of the pH of the buffer used as washing liquid on the recovery of verapamil and norverapamil. In all experiments, the same buffer was used for the conditioning and washing steps.
        As can be seen in Table 4, the washing of the DECs with pH 3.0 buffer causes the recoveries of the two analytes to decrease drastically. Lower recoveries were also obtained by using water instead of pH 7.4 buffer as

TABLE 3

Optimization of the Eluent Composition

Eluting liquid	Absolute recovery (%)	
(0.3 mL)	NV	V
ACN	22.4	18.2
CH3OH	80.1	81.1
CH3OH+ 0.1% AH	81.2	81.6
CH3OH+ 0.2% AH	87.3	88.3
CH3OH+ 0.4% AH	86.6	88.4

NV : Norverapamil   V : Verapamil   AH : 2-Aminoheptane
DEC : Bond Elut CN (50 mg)
Buffer added to the eluent: acetate buffer pH 3.0 (0.7 mL)
Analyte concentrations : 100 ng/mL

washing liquid. This can hardly be explained only by an increase of the ionization of the analytes since verapamil (pKa : 8.6) is already present essentially in protonated form at pH 7.4. The higher retention of the cationic analytes at pH 7.4 is probably related to an increased ionization of the free silanol groups at the surface of the cyanopropyl silica support. No significant changes in the analyte recoveries were obtained by use of a pH 9.2 borate buffer, which indicates that the analytes are not more strongly adsorbed in uncharged form than in cationic form on this kind of solid phase. Finally, the pH 7.4 phosphate buffer which had been used in preliminary experiments and in a similar automated SPE method [27], was selected as washing liquid.

TABLE 4

Optimization of the Washing Step

Washing	Absolute recovery (%)	
liquid	NV	V
B. pH 7.4 (phosphate)	88.1	88.5
B. pH 9.2 (borate)	87.5	81.3
B. pH 3.0 (acetate)	46.6	39.3
Water	49.0	70.4

NV. : Norverapamil   V. : Verapamil   B : Buffer
DEC : Bond Elut CN (50 mg)
Concentration of the drugs : 100 ng/mL
Conditioning liquids : 1. Methanol 2. Same as washing liquid
Eluents : 1. Methanol (0.3 mL) 2. pH 3.0 buffer (0.7 mL)

Type of solid phase

Table 5 shows the recoveries of verapamil and norverapamil obtained with aqueous solutions of the drugs by using different kinds of solid phases in the DECs.

Due to the difficulties to obtain a quantitative elution of the two analytes, i.e. a recovery greater than 90 %, from DECs filled with ordinary cyanopropyl silica, other kinds of sorbents were tested. As observed previously [27], an increase of the amount of sorbent in the cartridge did not give rise to significant changes in recoveries. However, as can be seen in Table 5, a slight increase in the recoveries of verapamil and norverapamil was obtained by using DECs packed with endcapped cyanopropyl silica (CN^{EC}), probably due to a better elution of these compounds from the DECs by reduction of their interactions

TABLE 5

Type of Sorbent Used in the DECs

Sorbent (mg)	Absolute recovery (%)	
	NV	V
CN 50	86.1	84.8
CN 100	83.1	79.8
CN 50 Endcapped(EC)	93.7	92.6
C18 50	93.0	89.6

NV: Norverapamil   V : Verapamil
Concentration of the drugs: 100 ng/mL
Eluent: CH3OH + 0.2% 2-Aminoheptane

with silanol groups. The analyte recoveries on C18 cartridges were about the same as those obtained with the endcapped cyano phase but the preference was given to the more polar phase, due its higher extraction selectivity.

Analyte-sorbent interaction can be affected by the sample matrix which may compete for the active sites of the support material and cause the adsorption of the analytes to decrease [32,33]. Therefore recoveries from plasma samples spiked with verapamil and norverapamil were studied immediately afterwards. Such plasma samples treated by the automated SPE procedure under the operational conditions selected above and applied on the DECs with the minimum dispensing flow rate (0.18 mL/min) [23] gave similar recoveries to those obtained with aqueous standard solutions. This seems to indicate that there are no significant effects from the matrix components on the adsorption and consequently on the recoveries of the analytes. The different SPE parameters optimized with aqueous solutions as described above can thus be used without modification for handling plasma samples.

TABLE 6

Absolute Recoveries of the Analytes

Conc. (ng/mL)	NV (%)	V (%)	G (%)
500	89	86	-
200	92	89	-
100	99	97	95
50	96	98	-
20	101	103	-
10	88	96	-
Mean	94	95	95

NV. : Norverapamil   V. : Verapamil   G. : Gallopamil

Volume of eluent

The last parameter to be optimized in the development of such an automated SPE procedure is the volume of eluent. In order to improve the detectability (LOD/LOQ) for the analytes in the present method without including any evaporation step, the minimum volume of solvent which still gives a satisfactory elution of these compounds was determined. Smaller volumes for the elution step could already be used with DECs containing 50 mg of sorbent instead of 100 mg [27], similar recoveries being obtained with both kinds of cartridges (cfr. Table 5).

Fig. 4 shows that a significant decrease of the recovery of verapamil was only obtained when the volume of eluent was lower than 0.20 mL. A volume of 0.24 mL of methanol containing 0.2 % of 2-aminoheptane was finally selected for the elution of the two analytes, norverapamil and verapamil having a similar behaviour. In order to obtain a final extract with an eluting strength comparable to that of the HPLC mobile phase while minimizing dilution, 0.41 mL of pH

3.0 buffer should then be passed through the DEC, giving the extract a total volume of 0.65 mL (concentration factor : 1.54).

Validation of the automated procedure

A typical chromatographic trace of a plasma extract containing verapamil and norverapamil (concentration : 10 ng/mL) is shown in Fig. 5B. Under the conditions selected for the HPLC separation, the mean capacity ratios (k') of norverapamil, verapamil and gallopamil were 3.4, 3.9 and 4.8, respectively (n =20).

Absolute recovery

Table 6 gives the absolute recoveries of the analytes at six different concentrations ranging from 10 to 500 ng/mL. The mean absolute recoveries for the two analytes were around 95 %. These absolute recoveries were calculated by comparing peak areas obtained from freshly prepared sample extracts with those found by direct injection of aqueous standard solutions at the same concentration [34], using the same autosampler.

Linearity

The calibration curves were constructed in the range 3-500 ng/mL (n=8), the therapeutic plasma levels of verapamil exceeding not 500 ng/mL [1]. Linear regression analysis made by plotting the analyte/internal standard peak area ratio (y) versus the concentration (x) in ng/mL gave the following equations :

norverapamil : $y = 0.0154 \, x + 0.0135$ $r^2 = 0.99999$
verapamil : $y = 0.0149 \, x + 0.0453$ $r^2 = 0.99996$

The linearity of the calibration curves is demonstrated by the good determination coefficients (r^2) obtained for the regression lines.

Reproducibility

As shown in Table 7, the precision of the bioanalytical method was estimated by measuring the within-day and between-day reproducibilities of the analytes at four concentration levels ranging from 10 to 200 ng/mL. Mean values around 2.3 % and 3.7 % were obtained, respectively.

Detectability

Limits of detection (LOD) and limits of quantitation (LOQ) were calculated from regression lines [35] obtained with calibration curves ranging from 2 to 100 ng/mL. The LOD for verapamil was equal to 1.0 ng/mL and its

TABLE 7

Reproducibility of the Automated Method

Within-day reproducibility			
Concentration (ng/mL)	n	Norverapamil R.S.D.(%)	Verapamil R.S.D.(%)
200	5	2.0	2.2
100	6	1.9	1.4
50	5	1.5	2.4
10	5	4.2	3.0
Mean :		2.4	2.3
S.D. :		1.2	0.7

Between-day reproducibility			
Concentration (ng/mL)	n	Norverapamil R.S.D.(%)	Verapamil R.S.D.(%)
200	5	2.4	2.2
100	5	3.0	1.9
50	5	3.9	4.1
10	5	5.6	6.3
Mean :		3.7	3.6
S.D. :		1.4	2.0

NV. : Norverapamil   V. : Verapamil   G. : Gallopamil

LOQ to 3.3 ng/mL. The LOD and LOQ for norverapamil were equal to 1.7 ng/mL and 5.8 ng/mL, respectively.

Selectivity

No endogenous sources of interference were observed at the retention times of the analytes. Typical chromatograms obtained with a blank plasma (A) and with a spiked plasma containing with 10 ng/mL of each drug (B) are presented in Fig. 5.
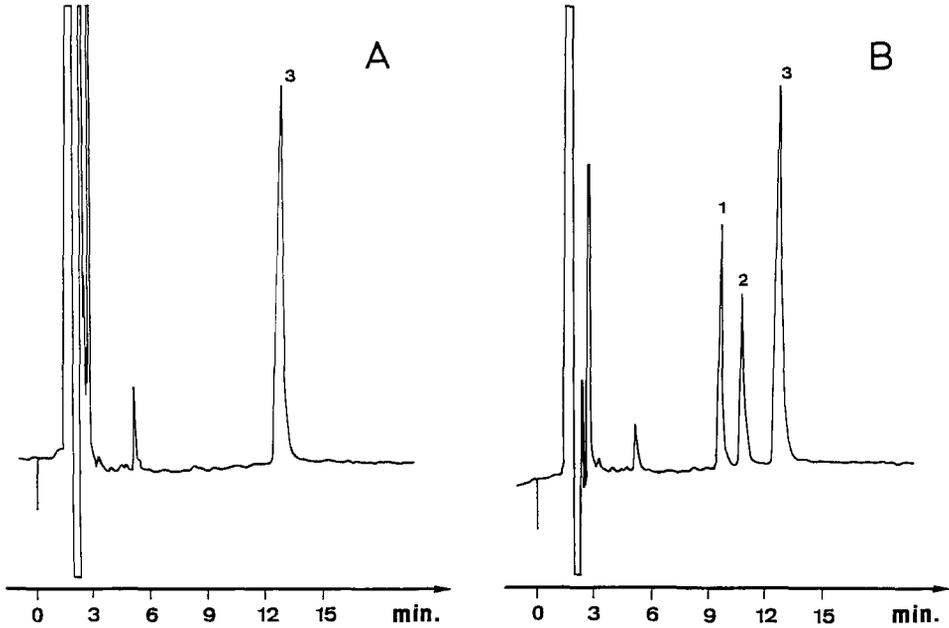
FIGURE 5

Typical chromatograms obtained by using SPE on DEC coupled to HPLC
Mobile phase :   pH 3.0 acetate buffer :   70
                     Acetonitrile :   30
                     2-Aminoheptane :   0.5
Solid phase : Superspher 100 RP-18 (4 μm)
Fluorescence detection : excitation, 275 nm; emission, 310 nm
DEC : Bond Elut CN endcapped (CN^{EC}; 50 mg).
Sample :   A : blank plasma
                 B : spiked plasma
Peaks :   1 : Norverapamil :   5.9 ng   (10 ng/mL)
              2 : Verapamil :   5.9 ng   (10 ng/mL)
              3 : Gallopamil [IS] : 57.8 ng   (98 ng/mL)

Application of the automated method

The fully automated method developed for the determination concentrations of both verapamil and norverapamil in human plasma has been applied successfully to more than 4600 analyses in the framework of bioavailability studies and has proved to be sensitive and rugged.

REFERENCES

[1] Singh, B.N., Ellrodt, G., and Peter T. Verapamil : A Review of its Pharmacological Properties and Therapeutic Use. Drugs, 15, 169, 1978.

[2] Cole, S.C.J., Flanagan, R.J., Johnston, A., and Holt, D.W. Rapid High-Performance Liquid Chromatographic Method for the Measurement of Verapamil and Norverapamil in Blood Plasma or Serum. J. Chromatogr., 218, 621, 1981.

[3] Hege, H.G. Gas Chromatographic Determination of Verapamil in Plasma and Urine. Arzneim-Forsch/Drug Res., 29 (II), 1681, 1979.

[4] McAllister, R.G., Tan, T.G., and Bourne, D.W.A. GLC Assay of Verapamil in Plasma : Identification of Fluorescent Metabolites after Oral Drug Administration. J. Pharm. Sci., 68, 574, 1979.

[5] Remberg, G., Ende, M., Eichelbaum, M., and Schomerus, M. Mass Spectrometric Identification of DL-Verapamil Metabolites. Arzneim-Forsch/Drug Res., 30 (I), 398, 1980.

[6] Giachetti, C., Poletti, P., and Zanolo, G. Analysis of Calcium Blocker Drugs in Plasma, HRGC and HPLC Analytical Conditions for Pharmacokinetic Studies. J. High Resol. Chromatogr., 10, 654, 1987.

[7] Harapat, S.R., and Kates, R.E. Rapid High-Pressure Liquid Chromatographic Analysis of Verapamil in Blood and Plasma. J. Chromatogr., 170, 385, 1979.

[8] Harapat, S.R., and Kates, R.E. High-Performance Liquid Chromatographic Analysis of Verapamil, II. Simultaneous Quantitation of Verapamil and its Active Metabolite, Norverapamil. J. Chromatogr., 181, 484, 1980.

[9] Jaouni, T.M., Leon, M.B., Rosing, D.R., and Fales, H.M. Analysis of Verapamil in Plasma by Liquid Chromatography. J. Chromatogr., 182, 473, 1980.

[10] Kuwada, M., Tateyama ,T., and Tsutsumi, J. Simultaneous Determination of Verapamil and its Seven Metabolites by High-Performance Liquid Chromatography. J. Chromatogr., 222, 507, 1981.

[11] Piotrovskii, Y.K., Rumianisev, D.O., and Metelitsa, V.I. Ion-Exchange High-Performance Liquid Chromatography in Drug Assay in Biological Fluids, II. Verapamil. J. Chromatogr., 275, 195, 1983.

[12] Hubert, Ph., Chiap, P., Ceccato, A., Bechet, I., Sibenaler-Dechamps, R., Maes, P., and Crommen, J. Determination of Verapamil and Norverapamil in Human Plasma by Liquid Chromatography : Comparison between a Liquid-Liquid extraction Procedure and an automated Liquid-Solid Extraction Method for Sample Preparation. J. Pharm. Biomed. Anal., 10, 937, 1992.

[13] Westerlund, D. Direct Injection of Plasma into Liquid Chromatographic Systems. Chromatographia, 24, 155, 1987.

[14] Lim, C.K. Sample Preparation for High-Performance Liquid Chromatography in the Clinical Laboratory. Trends Anal. Chem., 7, 340, 1988.

[15] McDowall, R.D., Doyle, E., Murkitt, G.S., and Picot, V.S. Sample Preparation for the HPLC Analysis of Drugs in Biological Fluids. J. Pharm. Biomed. Anal., 7, 1087, 1989.

[16] Huber R. and Zech K., Selective Sample Handling and Detection in High Performance Liquid Chromatography, part A, Chapter 2, eds., Frei R.W. and Zech K., Elsevier, Amsterdam, 1988, p. 81.

[17] Voelter, W., Kronbach, T., Zech, K., and Huber, R. A Simple High-Performance Liquid Chromatographic Precolumn Technique for Investigation of Drug Metabolism in Biological Fluids. J. Chromatogr., 239, 475, 1982.

[18] Frei, R.W. New Sample Handling Strategies in HPLC. Swiss. Chem., 6, 55, 1984.

[19] Hubert, Ph., Renson, M., and Crommen, J. A Fully Automated High-Performance Liquid Chromatographic Method for the Determination of Indomethacin in Plasma. J. Pharm. Biomed. Anal., 7, 1819, 1989.

[20] Lingeman, H., McDowall, R.D., and Brinkman, U.A.Th. Guidelines for Bioanalysis Using Column Liquid Chromatography. Trends Anal. Chem., 10, 48, 1991.

[21] McDowall, R.D., Pearce, J.C., and Murkitt, G.S. Liquid-Solid Sample Preparation in Drug Analysis. J. Pharm. Biomed. Anal., 4, 3, 1986.

[22] McDowall, R.D., Pearce, J.C., and Murkitt, G.S. Sample Preparation Using Bonded Silica : Recent Experiences and New Instrumentation. Trends Anal. Chem., 8, 134, 1989.

[23] Hubert, Ph., and Crommen, J. Automatic Determination of Indomethacin in Human Plasma Using Liquid-Solid Extraction on Disposable Extraction Cartridge in Combination with HPLC. J. Liq. Chromatogr., 13, 3891, 1990.

[24] Hubert, Ph., Chiap, P., and Crommen, J. Automatic Determination of Diltiazem and Desacetyldiltiazem in Human Plasma using Liquid-Solid Extraction on Disposable Extraction Cartridge coupled to HPLC - Part I : Optimization of the HPLC System and Method Validation. J. Pharm. Biomed. Anal., 9, 877, 1991.

[25] Hubert, Ph., and Crommen, J. Paper presented at the 2nd International Symposium on Pharmaceutical and Biomedical Analysis, York, U.K., April 4-7, 1990.

[26] United States Pharmacopeia XXII, Official Monographs, United States Pharmacopeial Convention, Rockville, MD, 1990, p. 1444.

[27] Hubert, Ph., Chiap, P., and Crommen, J. Automatic Determination of Diltiazem and Desacetyldiltiazem in Human Plasma using Liquid-Solid Extraction on Disposable Extraction Cartridge coupled to HPLC - Part II : Optimization of liquid-solid extraction. J. Pharm. Biomed. Anal., 9, 883, 1991.

[28] Hubert, Ph., and Crommen, J. Paper presented at the IVth International Symposium on Quantitative Luminescence Spectrometry in Biomedical Sciences, Ghent, Belgium, May 27-31, 1991.

[29] Musch, G., and Massart D.L. Isolation of Basic Drugs from Plasma using Solid-Phase Extraction with Cyanopropyl-Bonded Phase. J. Chromatogr., 432, 209, 1988.

[30] Marko, V., Soltés, L., and Radova, K. Polar Interactions in Solid-Phase Extraction of Basic Drugs by Octadecylsilanized Silica. J. Chromatogr. Sci., 28, 403, 1990.

[31] Law, B., Weir, S., and Ward, N.A. Fundamental Studies in Reversed-Phase Liquid-Solid Extraction of Basic Drugs; I : Ionic Interactions. J. Pharm. Biomed. Anal., 10, 167, 1992.

[32] Musch, G., Buelens, Y., and Massart, D.L. A Strategy for the Determination of Beta Blockers in Plasma using Solid-Phase Extraction in Combination with High-Performance Liquid Chromatography. J. Pharm. Biomed. Anal., 7, 483, 1989.

[33] Law, B., Weir, S., and Ward, N.A. Fundamental Studies in Reversed-Phase Liquid-Solid Extraction of Basic Drugs; III : Sample Matrix Effects. J. Pharm. Biomed. Anal., 10, 487, 1992.

[34] Buick, A.R., Doig, M.V., Jeal, S.C., Land, G.S., and McDowall, R.D. Method Validation in Bioanalytical Laboratory. J. Pharm. Biomed. Anal., 8, 629, 1990.

[35] Miller, J.C., and Miller, J.N., Statistics for Analytical Chemistry (R.A., Chalmers, and M., Masson, Ed.), Ellis Horwood,Chichester, 1984, p. 96.

SIMULTANEOUS DETERMINATION OF CLOMIPRAMINE AND ITS DEMETHYLATED METABOLITE IN PLASMA AND ERYTHROCYTES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

P. MARESCAUX[1], E. BELAN[2], N. HOUDRET[1],
M. GOUDEMAND[2], AND M. LHERMITTE[1,3]
[1]*Laboratoire de Biochimie Hôpital Calmette
rue du Pr. J. Leclercq
59045 Lille Cedex, France*
[2]*Service de Psychiatrie Centre Hospitalier Regional de Lille
avenue de Verdun
59045 Lille Cedex, France*
[3]*Laboratoire de Toxicologie
Faculté de Pharmacie
3 rue du Pr. Laguesse
59045 Lille Cedex, France*

ABSTRACT

Clomipramine and its N-demethylated metabolite, N-demethyl-clomipramine were determined simultaneously in plasma or in erythrocytes by a simple hexane-diethyl ether-step extraction procedure followed by reversed-phase liquid chromatography. Baseline separation was achieved by a 5-μm cyanopropylsilane column. The mobile phase consisted of 10mM phosphate buffer-methanol-acetonitrile (25:15:60, v/v/v), at a flow rate of 1.2 ml/min. The eluant was monitored by a UV detector operating at 220 nm. The inter- and intra assay coefficients of variation were within 3.6 and 6.0 % for clomipramine and 4.1 and 6.5 % for N-demethyl clomipramine, respectively. The lowest limit of detection of clomipramine and N-demethylated clomipramine was 5 ng/ml. The method is sensitive, specific and allows for routine analysis in therapeutic controls of patients treated by this antidepressant.

INTRODUCTION

The efficacy of tricyclic antidepressants for treating depression has been substantiated by more than 30 years of experience (1). The correlation between plasma levels of some tricyclic antidepressants and therapeutic effects suggests that measurement of plasma levels may provide valuable information in improving the clinical management of depressed patients (2).

The relationship between the plasma concentration of tricyclic antidepressants and their clinical effect on depressive symptoms is controversial (3). The drugs display marked interindividual differences in the steaty-state concentrations in serum (4), but monitoring therapeutic levels of these drugs is important, since the side effects : anticholinergic effects and cardiac toxicities, are quite common and mainly dose related (5).

Clomipramine is a tricyclic antidepressant medication widely used in western Europe. The drug after absorption, undergoes an important first-pass metabolism to N-demethyl-clomipramine which is pharmacologically active and participates in both therapeutic and unwanted effects (6). After reaching the systemic circulation, clomipramine is further biotransformed into N-demethyl-clomipramine, and both active principles are hydroxylated to metabolites which are further conjugated before being excreted in urine. Hydroxylation of parent drug and metabolite is under polymorphic genetic control by the same cytochrome P450 as debrisoquine and sparteine (6, 7).

Studies concerning blood concentrations of clomipramine and N-demethyl-clomipramine are still conflecting. Clomipramine is an antidepressant with a fairly narrow therapeutic effect. This fact combined, with a high-interindividual variability makes this drug candidate for blood concentration monitoring. Intraindividual variability could be also explained by different distribution in erythrocytes and it will be interesting to know the concentration of the drug in these cells.

A number of analytical methods have been used to determine clomipramine and N-demethyl-clomipramine in biological fluids (2, 8). The most commonly used techniques are based on separations by gas (6, 9-11) or liquid chromatography (5-7, 12-15).

Here we describe an HPLC method which allows the determination of clomipramine and N-demethyl-clomipramine, both in plasma and erythrocytes.

MATERIALS and METHODS

Reagents and chemicals

All chemicals were of analytical grade. Clomipramine, N-demethyl-clomipramine were kindly supplied by Ciba-Geigy laboratory (Rueil-Malmaison, France). Levalorphan, used as internal standard, was a gift from Hoffman-La Roche (Basel, Switzerland). HPLC-grade acetonitrile was obtained from FSA Laboratory, England and HPLC-grade methanol from Scharlau, Spain.

Sample collection and storage.

Blood samples were drawn into red-top vacutainer Tubes (Becton-Dickinson & co., France) and centrifuged within 2h of collection. Plasma and erythrocytes were separated and stored in propylene tubes at -20°C, until assayed

Standard Solutions and Internal Standard

A stock solution containing 2500 ng/ml of clomipramine and N-demethyl-clomipramine was prepared in methanol. The internal standard stock solution of levalorphan (10 μg/ml) was also prepared in methanol. The solutions were stored at -20°C until required. Plasma standard solutions of clomipramine and N-demethyl-clomipramine for the calibration curves were prepared by appropriate dilution of the stock clomipramine and N-demethyl-clomipramine solutions with drug-free plasma so that concentrations of 5, 10, 25, 50, 100, 250 and 500 ng/ml were obtained.

Chromatographic conditions

The HPLC system consisted of a Model 1090, equipped with an automatic injector and a diode array, connected to an HP 85b computer (Hewlett-Packard, Orsay, France), a T5C recorder (Ifelec, Courbevoie, France). Separation was performed on a 5 μm cyanopropyl silane column (4 mm ID x 250 mm) (Société SGE, France) using a mobile phase consisting of 10 mM potassium phosphate buffer (pH 7.0) - methanol - acetonitrile (25: 15: 60, v/v) and a column temperature of 40°C. The flow rate was 1.2 ml/min. The detector was set up at 220 nm.

Sample preparation

- 100 μl of the internal standard (10 μg/ml levalorphan) were introduced in a silanized centrifuge tube and evaporated to dryness under nitrogen at 30°C, 1 ml of plasma standard solutions at different concentrations (5, 10, 25, 50, 100, 250 and 500 ng/ml) or 1 ml of plasma from patients was added. The contents were extracted with 5 ml of a mixture of hexane and diethyl ether (4 : 1, v/v) on a mechanical shaker for 10 minutes and briefly centrifuged (3 000 g, 4°C). The organic layer was tranferred to a clean centrifuge tube and the other phase was again treated by 5 ml of the mixture of hexane and diethyl ether (4 : 1, v/v), after mechanical shaking and centrifugation, the two organic phases were mixed and evaporated to dryness under nitrogen at 30°C. The dried residue was reconstituted with 100 μl of mobile phase and a sample (25 μl) was injected onto the HPLC column.

- For the determination of clomipramine and N-demethyl-clomipramine in erythrocytes, 1ml of erythrocytes was added to a silanized centrifuge tube, containing 1 μg of levalorphan (internal standard), as previously described above. The contents were mixed with hexane-diethyl ether and extracted as previously described for the plasma.

Calibration Curve.

Peak-areas for clomipramine or N-demethyl-clomipramine and the internal standard levalorphan were measured and peak-area ratios (clomipramine or N-demethyl-

Table 1   Drugs tested for possible interference in the HPLC assay of
           clomipramine and N-demethyl clomipramine

Paracetamol	Oxazepam
Salicylic acid	Prazepam
Carbamazepine	Triazolam
Phenytoin	Amitryptilin
Valproic acid	Imipramine
Digoxin	Fluvoxamine
Theophyllin	Haloperidol
Clonazepam	Thioridazine
Diazepam	Thioproperazine

clomipramine/internal standard) were used for preparation of a calibration curve. The calibration curves were constructed by plotting plasma clomipramine or N-demethyl-clomipramine concentrations (x axis), expressed as ng/ml, versus peak-area ratios (y axis), using linear regression.

This line was then used to calculate the concentration of the drug in the unknown samples (plasma or erythrocytes).

Recovery

Extracts from plasma, prepared as described above, were compared with a direct assay of standards in methanolic solution. These relative recoveries were determined for two different concentrations. The absolute recoveries were also determined for these two different concentrations from extracts of plasma, treated using the procedure described above, except that the internal standard was omitted. All extraction sample residues were reconstituted in 100 μl of the solution of internal standard (1 μg/ml) in mobile phase. In this recovery analysis, levalorphan served as external standard.

Interferences

Interference from endogeneous material and from other drugs was researched. drugs were tested at concentration of 1000ng / ml (Table I).

RESULTS and DISCUSSION

Fig 1 shows the separation and quantitation of clomipramine and N-demethyl-clomipramine in human plasma, using levalorphan as internal standard. In the chromatograms, which were obtained after extraction of 1.0 ml of blank plasma, no additional peaks that could interfere with the determination of the drug, its metabolite and the internal standard are present. Fig 1A represents a chromatogram of blank plasma. Similar result was obtained after extraction of 1 ml of erythrocytes. Blank plasma samples
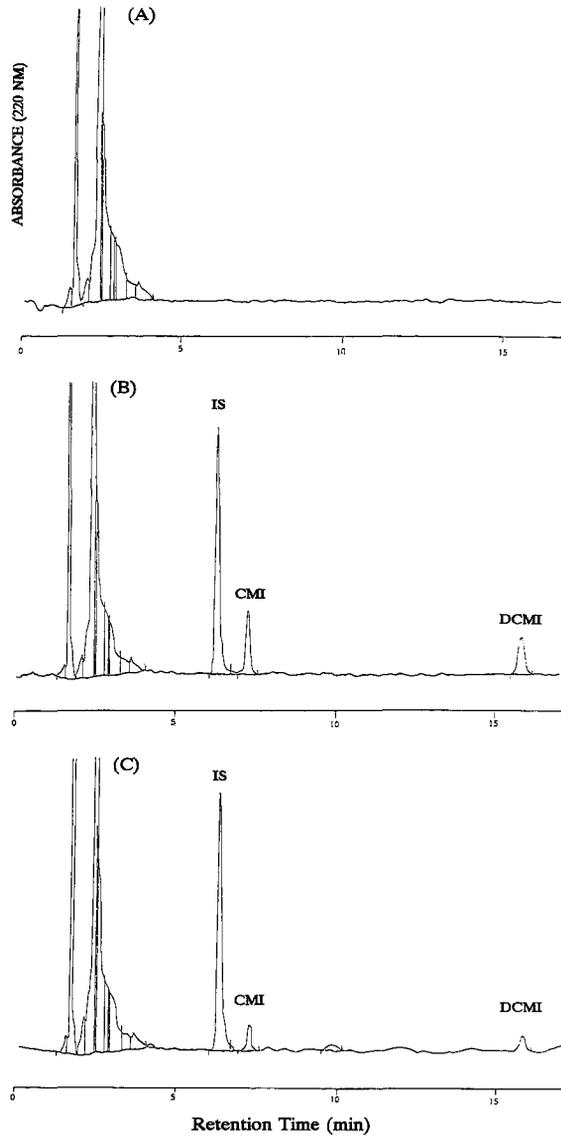
Figure 1 :

HPLC profiles of (A) drug-free human plasma ; (B) spiked drug-free human plasma (1 ml) containing 100 ng clomipramine and N-demethyl-clomipramine and 1000 ng/ml internal standard ; (C) human plasma containing 38 ng/ml clomipramine and 27 ng/ml N-demethyl-clomipramine - Peak CMI = clomipramine ; DCMI = N-demethyl-clomipramine ; IS = internal standard.

and blank erythrocytes sample from 30 subjects were analysed and no plasma or erythrocyte endogeneous peaks, co-eluting with the internal standard, the drug or the metabolite, were detected. Fig 1B is a chromatogram obtained after extraction of 1.0 ml of plasma spiked with 100 ng/ml of clomipramine and N-demethyl-clomipramine. The retention times for the internal standard, clomipramine, and N-demethyl-clomipramine were 6.30, 7.30 and 15.80 min, respectively. Fig 1C shows a chromatogram obtained after extraction of 1.0 ml of plasma from a patient treated by clomipramine.

The calibration curves for clomipramine and N-demethyl-clomipramine were linear over the concentration range of 5 to 500 ng/ml with the square of correlation coefficient (r^2) greater than 0.99. The typical linear relationship for the calibration curve can be expressed by the equations : $y = 0.0033 \, x - 0.004$, ($r^2 = 0,997$), in standard solution and $y = 0.0023 \, x + 0.015$, ($r^2 = 0,995$), in plasma solution for clomipramine and $y = 0.0029 \, x - 0.016$, ($r^2 = 0,996$), in standard solution and $y = 0.0021 \, x + 0.015$, ($r^2 = 0,996$), in plasma solution for demethyl-clomipramine, respectively.

Calibration curves in plasma showed good linearity between peak-area ratios and concentrations from 5 to 500 ng/ml, and the present method is able to detect 5 ng/ml of clomipramine and N-demethyl-clomipramine

Within-run precision (n=5) and day to day reproducibility (n=5), determined by this method were 3.6% and 6.0% for clomipramine and 4.1 and 6.5 % for N-demethyl-clomipramine, respectively, at a concentration of 100 ng/ml.

Recovery of clomipramine and its metabolite was estimated by comparing the peak areas of clomipramine or N-demethyl-clomipramine and the internal standard with those obtained by direct injection of the pure standards of clomipramine or N-demethyl-clomipramine and the internal standard. The mean recovery for clomipramine and for N-demethyl-clomipramine (n = 5) from plasma sample was 85.0 and 90.3 % at 100 ng/ml respectively.

Absolute recovery of the drug and its metabolite was estimated by using levalorphan as external standard. The mean recovery for clomipramine and for N-demethyl-clomipramine (n= 5) from plasma sample was 66.7 and 74.2% at 100 ng/ml, respectively.

Plasma samples or erythrocytes samples stored at -20°C for up to 2 months showed no signs of decomposition and practically the same concentration values were obtained (n = 6). This suggests that clomipramine and its metabolite are stable under these storage conditions, for at least 2 months.

Possible interference by other antidepressants and other drugs at their therapeutic concentrations was evaluated. Commonly used drugs tested (Table I) did not interfered with the assay

The assay is shown to be selective, without interferences from endogeneous materiel and from other drugs commonly used in therapeutic treatment.

The assay is also used for the determination of clomipramine and N-demethyl-clomipramine in erythrocytes.

The method has been applied to many patient samples and is being used routinely in the laboratory for monitoring therapeutic levels. The drug and its metabolite are determined using a simple procedure.

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REFERENCES.

1. Goodman W. K. and Charney D. S.
    J. Clin. Psychiatry. **40**, 6, 1985.

2. Scoggins B.A., Maguire K.P., Norman T.R. and Burrows G.D.
    Clin. Chem. **26**, 805, 1980.

3. Montgomery S.A., McAuley R., Montgomery D.B., Dawling S. and Braithwaite R.A.
    Postgraduate Medical Journal, **56** (suppl I), 130, 1980.

4. Preskorn S. H., Dorey R. C. and Jerkovich G. S.
    Clin. Chem. **34**, 822, 1988.

5. Matsumoto K., Kanba S., Kubo H., Yagi G. and Yuki H.
    Clin. Chem. **35**, 453, 1989.

6. Balant-Gorgia A.E., Gex-Fabry M. and Balant L.P.
    Clin. Pharmacokinet. **20**, 447, 1991.

7. Balant-Gorgia A.E., Balant L.P., Genet C., Dayer P. and Aeschliman J.M.
    J. Clin. Pharmacol. **31**, 449, 1986.

8. Norman T.R. and Maguise K.P.
    J. Chromatogr. **340**, 173, 1985.

9. Ninci R. and Sgaragli.
    J. Chromatogr. **381**, 315, 1986.

10.   Sioufi A., Pommier F. and Dubois J.P.
    J. Chromatogr. **428**, 71, 1988.

11.   Balant-Gorgia A.E., Balant L.P. and Garrone G.
    Ther. Drug Monitoring. **11**, 415, 1989.

12.   Sutfin T.A., D'Ambrosio R. and Jusko W.
    Clin. Chem. **30**, 471, 1984.

13.   Visser T., Oostelbus M.C.J.M. and Toll P.J.M.M.
    J. Chromatogr. **309**, 81, 1984.

14.   Spreux-Varoquaux O., Morin D., Advenier C. and Pays M.
    J. Chromatogr. **416**, 311, 1987.

15   Diquet B., Thomaré P., Becquentin M.and Diviné C.
    Biomed. Chromatogr. 7, 59, 1993.

AUTOMATED DETERMINATION OF AN ANGIOTENSIN II RECEPTOR ANTAGONIST, CGP 48 933, IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

A. SIOUFI, F. MARFIL, AND J. GODBILLON

Biopharmaceutical Research Center
Laboratoires CIBA-GEIGY
B.P. 306
92506 Rueil-Malmaison, France

ABSTRACT

A fully automated high-performance liquid chromatography method with fluorimetric detection is described for the determination of CGP 48 933 in human plasma. Liquid-solid extraction was performed automatically on C8 reversed phase column using the Gilson ASPEC system. The on-line chromatography was performed on a ODS Hypersil C18 5 μm column. The mobile phase, acetonitrile- pH 2.8 phosphate buffer (50:50, v/v) was used at a flow rate of 1.3 ml/min. The fluorimetric excitation and emission wavelengths were set at 265 and 378 nm, respectively. The limit of quantitation of CGP 48 933 was 11.5 nmol per litre of plasma.

INTRODUCTION

CGP 48 933 is a new class of antihypertensive compound (Figure 1) which acts through blockade of the angiotensin II receptors. The renin-angiotensin system which plays an important role in the regulation of blood pressure, may be blocked either by angiotensin converting enzyme inhibitors or at the angiotensin II receptors.

To investigate the pharmacokinetics of CGP 48 933 in humans, a high performance liquid chromatography method was developed using a structural analog as internal standard (Figure 1). Previously, a HPLC method was described for the determination of another compound with similar activity (1).

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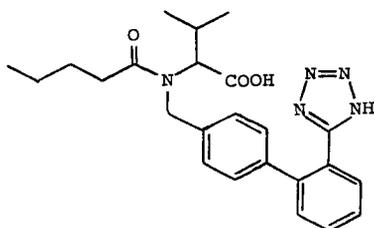
CGP 48 933

Mol. wt. = 435.52
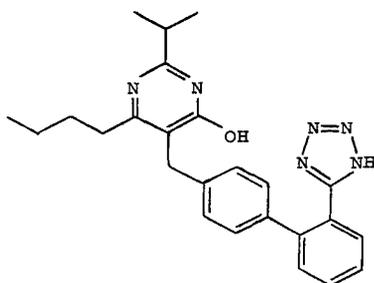
CGP 48 791
Internal standard

Mol. wt. = 428.55

Figure 1 : Chemical structures.

MATERIALS

CGP 48 933 and the internal standard were supplied by Ciba-Geigy (Basle, Switzerland).

The reagents were all of analytical grade. Acetonitrile for HPLC, methyl alcohol for UV and potassium dihydrogenphosphate were purchased from Carlo Erba France. 0.1 N hydrochloric acid, 0.1 N sodium hydroxide and 85 % phosphoric acid were from Merck (Darmstadt, Germany). Water was purified with a Millipore Milli-Q system (Millipore, France).

Disposable extraction columns (DECs) of 1 ml capacity containing 100 mg C8 reversed phase(Bond-Elut, Analytichem International, Varian, France) were used for liquid-solid extraction.

Apparatus

The chromatographic equipment was as follows :

. A fully automated analytical system (ASPEC, Gilson, Villiers-le-Bel, France), based on automated liquid-solid extraction and injection combined with HPLC.

. A solvent delivery system, pump model 302 associated with a
  manometric module model 802C (Gilson).

. A fluorimetric detector Hitachi, model 1050 (Merck,France).
  The excitation and emission wavelengths were set at 265 and
  378 nm, respectively.

. A data pool workstation NEC 810 (Waters, Millipore, France)
  which performed integration, recording and storage of the
  data.

Chromatographic conditions

     A pre-packed column ODS Hypersil C18 (150 mm x 4.6 mm I.D.,
5 μm particle size, Hewlett-Packard, France) was used for the
separation. To protect this column, a guard column Supelguard
LC-8, 20 mm x 4.6 mm (Supelco, France) was installed.

     The buffer of the mobile phase was prepared by dissolving
1.36 g of potassium dihydrogenphosphate in one litre of
distilled water. The final pH 2.8 was adjusted with thirteen
drops of 85 % phosphoric acid. The mobile phase (pH 2.8
phosphate buffer-acetonitrile (50:50, v/v) was used at a flow
rate of 1.3 ml.

Standard and validation solutions

     Standard and validation solutions were obtained by
dissolving 2.29 μmol (1 mg) of CGP 48 933 in 50 ml of methanol
and by successive dilutions of the master solution in methanol.
The plasma standard concentrations of CGP 48 933 ranged from
11.5 nmol/l to 4600 nmol/l.

     2.33 μmol (1 mg) of internal standard (I.S.) was dissolved
in 50 ml methanol.

                              METHODS

Sample extraction

     100 mg C8 DECs were placed on the rack of the ASPEC. An
aliquot of the I.S. solution was introduced into a 5 ml
polypropylene tube and evaporated to dryness. After addition and
mixing of 1 ml of plasma, the tube was placed on the rack of the
ASPEC system.

     300 μl of the eluate were dispensed through the 50 μl
injection loop.

     Each plasma sample was prepared separately during the
chromatography of the previous sample.

     CGP 48 933 and I.S. were found to be partly adsorbed on the
needle of ASPEC. A preliminary washing of the needle with
methanol and 0.1 N NaOH before each sample preparation was
necessary to avoid cross-contamination.

All the following operations on the samples were automatically performed :

Successive steps	Liquid dispensed on the DEC	Dispensing flow rate μl/sec (ASPEC code)	Pressurising air volume (μl)
DEC conditionning	1. Methanol 2 ml 2. 0.1 N HCl 2 ml	100 (code 5)	50
Sample distribution	1 ml 0.1 N HCl was added to 1 ml plasma and the 2 ml were dispensed on the DEC	6 (code 1)	1000
Washing	1. 1 ml KH_2PO_4 pH2.8 2. 0.5 ml KH_2PO_4/ CH3CN (70/30,v/v)	50 (code 4)	1000
Elution	2 ml of mobile phase (50:50, v/v)	50 (code 4)	1000

Calibration curve

Calibration samples were prepared by adding aliquots of various methanolic solutions of the compound to plasma. The calibration curves were established from the peak height ratio compound/I.S. plotted versus the concentrations of CGP 48 933 in the samples. Their equations were calculated by the least-squares method using weighted linear regression with a weighting factor of $1/(concentration)^2$.

RESULTS

As shown in Figure 2, CGP 48 933 and internal standard were well separated from human plasma components. No endogenous peaks interfered with the detection of CGP 48 933 and I.S. and under the chromatographic conditions the retention times of the compounds were 5 and 8 min, respectively.

An example of a calibration graph is given in Figure 3. Plasma samples containing different concentrations of CGP 48 933 were repeatedly analysed either on the same day in six replicate (within-day precision) or on five consecutive days (between-day precision).The results obtained with the procedure described are given in Tables 1 and 2, where the recovery (%) was calculated as the given concentration/found concentration multiplied by 100.
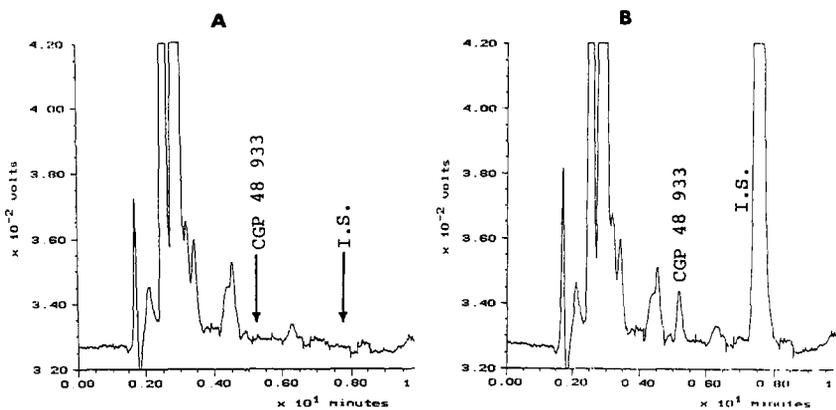
Figure 2 : Example of chromatograms obtained from an extract of
1 ml human plasma.
(A) : 1 ml drug-free plasma.
(B) : 1 ml plasma spiked with 23 pmol (10 ng) of
CGP 48 933 and 1167 pmol (500 ng) of I.S.
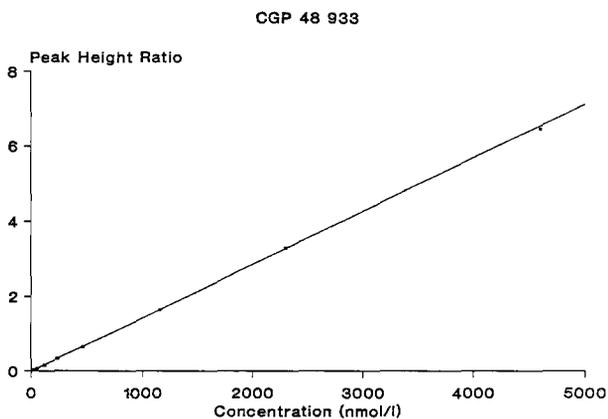
Figure 3 : An example of calibration curve of CGP 48 933.
Slope : 0.00142.
Intercept : − 0.00174.
Correlation coefficient : 0.999.

TABLE 1

Within-day reproducibility and accuracy of
the determination of CGP 48 933 in human plasma.

Given (nmol/l)	11.5	23.0	920	3450
Recovery (%)	109	99.8	96.9	101
	98.1	98.0	98.3	97.8
	98.1	95.5	97.9	98.2
	101	108	97.6	98.5
	96.9	91.3	96.8	98.6
	93.2	95.5	98.9	97.1
Mean (%)	99.4	98.0	97.7	98.5
CV (%)	5.4	5.8	0.8	1.3
SD	5.3	5.7	0.8	1.3

TABLE 2

Day-to-day reproducibility and accuracy of
the determination of CGP 48 933 in human plasma.

Given (nmol/l)	11.5	23.0	57.5	920	3450
Day of analysis	Recovery (%)				
2	102	103	100	98.5	96.2
3	100	96.2	93.6	95.3	97.8
4	104	92.9	97.6	98.0	102
5	100	99.1	96.6	98.6	101
8	109	99.5	97.2	99.2	93.2
Mean (%)	103	98.1	97.0	97.9	98.0
CV (%)	3.6	3.9	2.4	1.6	3.7
SD	3.7	3.8	2.3	1.5	3.6

The limit of quantitation (coefficient of variation < 10 %) was calculated from the results in Table 1 and was 11.5 nmol/l of plasma.

The standard and validation solutions of CGP 48 933 and the I.S. solution were stable for at least three weeks at +4°C. This was determined by injecting daily aliquots of internal standard and compound solutions : as regards to the peak height, the results obtained over three weeks were compared with those obtained on the first day.

For a plasma sample spiked with a given amount of CGP 48 933 and I.S., a similar peak height was observed for the two compounds with extraction performed either immediately after preparation or 12 hours later. This indicated that there was no degradation of CGP 48 933 and the I.S. in the diluted plasma samples left at room temperature on the rack of the ASPEC system for several hours.

DISCUSSION

Various DEC's sorbent were tested. The extraction recoveries and the separation of the compounds from the plasma components were explored.

With 100 mg C2 DECs, the extraction recoveries were variable depending on the batch of the DECs. With 100 mg C8 or C18 DECs, the extraction recoveries were found more reproducible from batch to batch and were around 90 %.

These recoveries were similar to those of others studies (2, 3) which reported extraction recoveries from DECs around 90 %.

It appeared that high recoveries were required to obtain a good reproducibility over the time and the various batches of DECs.

A previous manual method for the plasma determination of CGP 48 933 using DECs had been developed in our laboratory. The results obtained with the two HPLC methods were compared. The limit of quantitation with a coefficient of variation inferior to 10 % was improved by 2 with the ASPEC system and the precision and accuracy were slightly better as shown in Table 3.

Given (nmol/l)	11.5	23.0	23.0
Day of analysis	Recovery (%)		
	ASPEC System		Manual
2	102	103	95.1
3	100	96.2	105
4	104	92.9	104
5	100	99.1	98.5
8	109	99.5	93.7
Mean (%)	103	98.1	99.7
CV (%)	3.6	3.9	5.6
SD	3.7	3.8	5.6

C
o   8000
n
c   6000   ☐ B
                                                         Χ C
n   4000
m
o   2000
l
/   0
l
         0   5   10   15   20   25

                        Time (h)
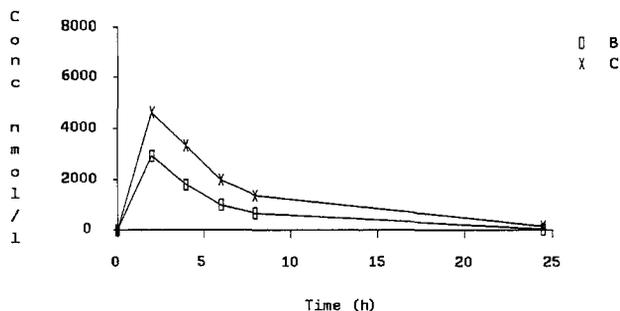
Figure 4 : Mean plasma concentration-time profiles after oral
           administration to six volunteers of either 40 mg (B)
           or 80 mg (C) of CGP 48 933.

APPLICATION

    This method was applied to plasma samples from volunteers
given a single oral doses of CGP 48 933. The mean plasma
concentration-time profiles are shown in Figure 4. At sampling-
time 24 h, the lowest concentration observed was three fold the
limit of quantitation. So the sensitivity of this method was
suitable for pharmacokinetic investigation.

CONCLUSIONS

    An automated HPLC method has been developed and validated
to quantify CGP 48 933 in plasma samples. The sensitivity of
this method was improved compared to a manual method and was
suitable to study the phamacokinetics of the compound.

REFERENCES

1. R. H.-Y. Chiou, M.-W. Lo, J. Chromatogr., 581: 165-170
(1992).

2. M.C. Rouan, J. Campestrini, V. Le Clanche, J.B. Lecaillon,
J. Godbillon, J. Chromatogr., 573: 65-68 (1992).

3. G. Hotter, G. Gomez, I. Ramis, G. Bioque, J. Rosello-Catafau,
E. Gelpi, J. Chromatogr., 607: 239-243 (1992).

DETERMINATION OF PIROXIMONE IN PLASMA AND URINE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

N. BERNARD[1], M. LUSH[2], J. PROBITTS[2],
N. FERRY[1], G. CUISINAUD[1], AND J. SASSARD[1]

[1]Department of Physiology and Clinical Pharmacology
URA CNRS 1483, Faculty of Pharmacy
8 Avenue Rockefeller
F-69373 Lyon Cedex 08, France
[2]Merrell Dow Research Institute
Winnersh Research Centre
Reading Road
Winnersh, Berkshire, RG11 5HQ, United Kingdom

ABSTRACT

A reversed-phase high-performance liquid chromatographic (HPLC) method with UV detection was developed for quantifying piroximone (PI) in plasma and urine. A solid phase extraction of PI simultaneously with an internal standard with an average recovery higher than 86% was needed to avoid endogenous interferences especially in urines from patients with reduced renal functions. The sensitivity limit was 2 ng/ml in plasma and 100 ng/ml in urine. The method was reproducible with intra- and inter-assay coefficients of variations below 7 %. This method was applied to the determination of plasma and urine levels during a pharmacokinetic study in healthy subjects and renally impaired patients. It was found suitable to follow the concentrations until 24 h after a single intravenous infusion of 0.5 mg/kg body weight of piroximone.

INTRODUCTION

Piroximone, 4-ethyl-1,3-dihydro-5-(4-pyridinyl-carbonyl)-2H imidazol 2 one, MDL 19.205 (PI) is an imidazole derivative with inotropic and vasodilator properties used in congestive heart failure.

Piroximone
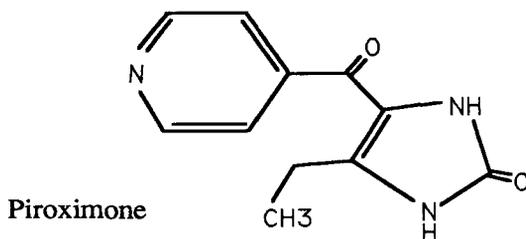
The pharmacokinetic of PI has been studied in healthy subjects as well as in patients with heart failure at dosage ranging from 0.2 to 2 mg/kg after IV or oral administration(1-2). In man, metabolites have not been identified, and urinary excretion of native drug amounts to 67 % of administered drug in the first hours following administration. In rat(3) metabolites : isonicotinic acid and hydroxypiroximone never exceed 15% of amount excreted. The aim of this study was to provide an analytical method sensitive and specific enough to follow in humans the plasma and urine levels of PI after a single bolus of 0.5 mg/kg body weight. Previous analytical methods(4-5) did not distinguish PI from interferences often encountered in plasma and urine from renally impaired patients and were found insufficiently specific in our hands. The present report describes an HPLC technique with improved sensitivity and specificity for the simultaneous determination of PI in plasma and urine.

MATERIALS.   METHODS

Chemicals

PI and internal standard (IS), 4-(3,4-dimethoxy-benzoyl)-1,3-dihydro-5-methyl-2H-imidazol-2-one; MDL 82.261 were supplied by Merrell Dow Laboratories. Methanol was purchased from Carlo Erba, Milano, Italy. Phosphoric acid, potassium dihydrogenphosphate and disodium hydrogenphosphate, dodecahydrate were purchased from Merck. All chemicals were of analytical grade. Columns for solid extraction : Bond Elut SPE ® 200mg/3ml from Analytichem International were distributed by Varian. Human plasma for laboratory use was obtained from CTS Lyon-Beysnot.

Plasma solutions were prepared by dissolving 10 mg of PI in 100 ml human plasma to obtain a 100 µg/ml concentration. Dilutions of 5, 1, 0.5, 0.1, 0.05, 0.01, and 0.002 µg/ml used for calibration and control were made by diluting previous solutions in the same unit of human plasma. Urine solutions were prepared in the same manner to obtain 100, 50, 10, 5 and 1 µg/ml concentrations. All standard solutions were frozen at least one week prior the assay under 2 ml volume in the conditions used for the samples of the pharmacokinetic study, that is −20°C, to avoid differences in extraction rate observed between fresh and frozen samples. Quality controls of plasma spiked with PI (50 and 500 ng/ml) and urine (5 µg/ml and 50 µg/ml) were stored from the beginning of the study in the same conditions. Solution of Internal Standard (100 µg/ml) was made by dissolving 10 mg of MDL 82.261 in 1 ml sodium hydroxide 1M in a volumetric flask and further completed to 100 ml with water. This solution was found stable only when stored at 4°C in darkness for a maximum of two weeks. Working solutions of IS were made just before extraction.

Chromatographic Conditions

Reversed phase HPLC was performed at 22°C (air conditioned) using a high pressure pump, 414 model, equipped with two pulse damper 810 model (Kontron, Zürich, Switzerland), an injector : autosampler model MSI 660, equipped with a 90 µl loop (Kontron), two guard and analytical columns (30+150 x 4.6 mm) filled with Spherisorb ODS2 C18, 5 µm (Interchim, Montluçon France). The absorbance of the eluent was monitored by use of a spectrophotometer, model Uvikon 432, with wavelength set at 320 nm (4 nm slides) and equipped with a 8 µl flow cell with 5 mm pathlength (Kontron). The whole system was managed by a central station model MT2 450 (Kontron) for interfacing all the peripherics and recording of chromatograms.

Optimal separation of compounds was obtained using a mobile phase consisting of disodium hydrogenphosphate 0.04 M and potassium dihydrogenphosphate 0.026 M, (the pH of which is adjusted to pH = 3,2 by addition of phosphoric acid 45 N), methanol, 70/30, v/v.

Extraction Procedure

For their conditioning, SPE columns placed on 10 ml polyethylene centrifuge tubes were twice rinsed with 0.5 ml methanol then 1 ml water. Centrifugation at 1000 rpm during 5 min was processed at each step of elution.

For plasma separation, on a conditioned Bond Elut column were applied 500 µl plasma (or standard or quality control) spiked with 1000 µl IS (0.50 µg/ml). Centrifugation at 2000 rpm was allowed during five minutes. Columns were washed two times with 1 ml of water followed by a 5 min centrifugation at 2000 r.p.m. Compounds were eluted in a conical glass tube with two 0.5 ml methanol fractions and the eluate was evaporated until dryness in a 37°C water-bath, under a stream of

nitrogen delivered by Pasteur pipettes in each tube. Residue was dissolved in 250 µl of mobile phase by vortexing process during 20 seconds. This solution is transferred into an Eppendorf microtube and 90 µl injected in the chromatograph, with a mobile phase flow of 1 ml/min.

For urine separation, on a conditioned Bond Elut column were applied 500 µl urine (or standard or quality control) spiked with 1000 µl IS (5 µg/ml). Centrifugation at 2000 r.p.m. was allowed during five minutes. Columns were washed two times with 1 ml water followed by a 5 min centrifugation at 2000 r.p.m. Compounds were eluted in a polyethylene tube with two 0.5 ml methanol fractions and the eluate was diluted with 2 ml of the buffer disodium hydrogenphosphate 0.04 M / potassium dihydrogenphosphate 0.026 M, (pH = 3,2) to obtain the composition of the mobile phase. This solution was transferred into an Eppendorf microtube and 90 µl injected in the chromatograph, with a mobile phase flow of 1 ml/min.

Calculations

The PI concentrations were determined in biological samples by means of the ratio of the peak height of PI to the IS's one and calculated from the standard ratio determined with the spiked samples. As six determinations of the ratio are made at three different concentrations in each batch, the correlation is obtained by linear regression between ratio and concentration. Multiple regression analysis (Statgraphics STSC Inc.software) is performed with the reverse of concentration as weight. Correlation coefficient was always higher than 0.990. In each analytical run are introduced spiked samples (10, 100, 1000 ng/ml of plasma and 1, 10, 100 µg/ml of urine in duplicate) to determine the standard ratio and 4 quality controls (50 and 500

ng/ml of plasma and 5 and 50 µg/ml of urine in duplicate) to assess the reproducibility of the method.

RESULTS AND DISCUSSION

Preliminary assay of existing methods (4-5) revealed satisfactory when applied only to the plasma of healthy volunteers, but in our study, separation from endogenous interferences especially in urine or even in plasma was not sufficient. Indeed, patients included in this pharmacokinetic study exhibited reduced renal functions and received several comedications : acebutolol, allopurinol, aténolol, diltiazem, enalapril, furosemide, isradipine, lorazepam, nicardipine, nitrendipine, pindolol, pipemidic acid, prazosin, prednisolone, simvastatin, trazodone). Therefore exogenous and endogenous substances are at higher levels in plasma and in urine of renally impaired patients than in healthy volunteers. In addition in this study dosage was weak (0.5 mg/kg) and current kinetic studies require long time follow-up to ascertain the terminal part of the curve when concentrations are near the minimum quantity level. For these reasons specificity and sensitivity were enhanced by research and development of a chromatographic system and of an extraction procedure. The finalized method only is described here.

Chromatographic Conditions

The main chromatographic characteristics are in table 1.

This parameters table shows a large resolution of the two compounds with a good quality of peaks in the chromatographic conditions chosen (mobile phase: methanol/buffer pH = 3.2 (30/70), flow : 1 ml/min; column : Spherosil C8, 5 µm (150x4.6 mm); injected volume : 90 µl; detection UV wavelength : 320 nm).

TABLE 1

Chromatographic Characteristics of a Mixture of Piroximone (1 μg/ml) and Internal Standard (0.5 μg/ml) in Mobile Phase.

PARAMETERS	COMPOUND	
	PIROXIMONE	INTERNAL STANDARD
Retention time (min)	4.82	10.05
Capacity factor (k')	1.41	4.03
Nr of theoretical plates (m^{-1})	19647	40900
Theoretical plate height (μm)	12.70	6.00
Asymetrical factor (As)	1.02	1.31
Resolution factor (Rs)	5.00	

PI was the main compound observed in plasma as well as in urine of healthy volunteers and patients. These results are in accordance with recent pharmacokinetic studies(6). None of the compounds used as comedications were observed in this conditions.

Figure 1(a and b) shows chromatograms of extracts obtained from plasma (spiked with 500 ng/ml IS) and urine (spiked with 50 μg/ml IS) from healthy subject 1 hour after administration of PI (0.5 mg/kg). In this subject the values were 992 ng/ml and 13 μg/ml for plasma and urine concentrations respectively.

Extraction

The solid phase extraction procedure was highly efficient. For plasma extraction procedure, the extraction efficiency has been expressed by the ratio of peak heights obtained for plasma standards (500 ng/ml)
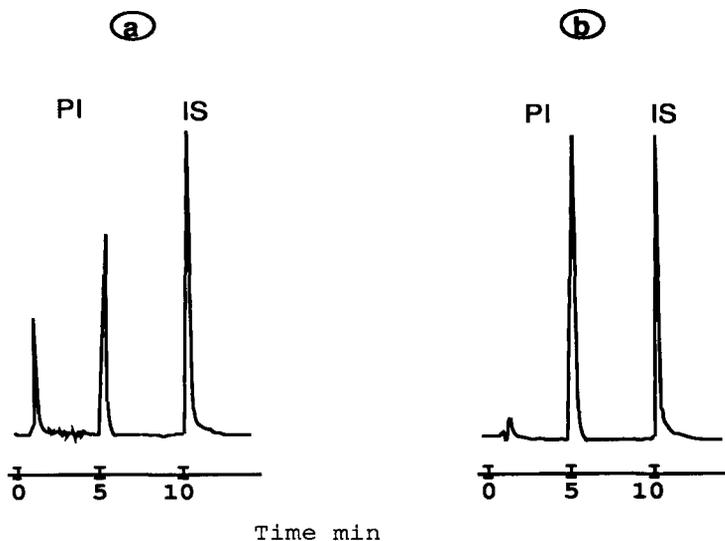
FIGURE 1. Chromatograms obtained with PI and IS in plasma (a) and urine (b) from patients 1 hour after I.V. administration of a 0.5 mg/kg dose of PI. (Full scale of the ordinate is 0.01 absorbance units for plasma and 0.1 absorbance units for urine).

to the peak height of pure solutions at the same concentrations. The extraction rates expressed as % (X ± SD) obtained for n = 10 determinations were found at 88.9% ± 3.1% for PI and 96.9% ± 2.8% for IS. For urine extraction procedure, the extraction rates expressed as % (X ± SD) obtained for n = 10 determinations (at 50 μg/ml) were found at 86.3% ± 3.6% for PI and 99.3% ± 3.2% for IS.

Characteristics of the Method

The linearity was studied in the range of expected concentrations during the kinetic study with samples spiked with PI at seven concentrations (extracted eight
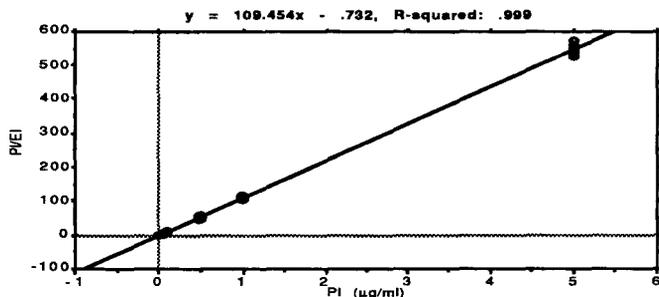
FIGURE 2. Linearity in plasma for PI concentrations
(n = 8) from 2 to 5000 ng/ml.

times) from 2 to 5000 ng/ml for plasma and at five con-
centrations (extracted eight times) from 1 to 500 µg/ml
for urine. The ratios of peaks height were expressed as
a function of PI concentrations. A test of linearity was
applied and regression line obtained for plasma
(r^2= 0.999) or urine(r^2= 0.996) are in figures 2 and 3.
In both cases the low intercept ascertain of the purity
of peaks measured.

      For reproducibility estimation, the coefficients
of variation intra- and inter-assay, expressed as SD %
of the value, were determined from quality controls
(biological samples spiked with PI at concentrations 50
and 500 ng/ml for plasma and 5 and 50 µg/ml for urine),
average of 10 determinations in the same run or one
determination on ten different days.

      Plasma and urine quality controls stored at -20°C
for up to 9 months showed no signs of decomposition
suggesting that PI is stable under these storage
conditions.

      The sensitivity limit is determined as the
quantity of extracted and injected compound which, in
the experimental conditions, is responsible of a peak
height 2 fold the residual noise. It was found in our
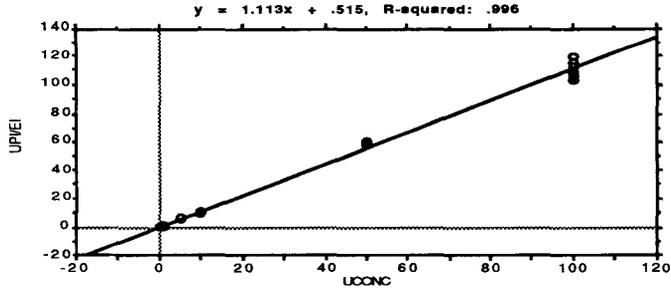
FIGURE 3. Linearity in urine for PI concentrations (n = 8) from 1 to 500 µg/ml.

TABLE 2

Intra- and inter-assay reproducibility of the method.

	added	Plasma recovered	added	Urine recovered
Intra	50	49,1 ± 1,9(3,9%)	5	5,3 ± 0,1(1,6%)
assay	500	491,5 ± 12,8(2,6%)	50	51,2 ± 0,6(1,1%)
Inter	50	48,0 ± 3,4(7,1%)	5	5,2 ± 0,2(3,1%)
assay	500	472,9 ± 15,1(3,2%)	50	51,6 ± 1,2(2,3%)

hands at 2 ng/ml of plasma and 0,1 µg/ml of urine (or less, depending on the dilution of urine).

The analytical technique is suitable for the determination of plasma samples in the range of 5 to 5000 ng/ml and urines in the range 1 to 100 µg/ml. This method was successfully applied to the determination of PI levels in plasma and urine of healthy and renally impaired patients after a single IV administration of

0.5 mg/kg of PI. Neither endogenous nor exogenous
compounds finally interfered with the detection of PI in
plasma or urine even in patients. No metabolites could
be identified.

ACKNOWLEDGMENTS

REFERENCES

1 - HAEGELE, K.D., BELZ, G.G., MEINICKE, T.T.,
     SCHECHTER, P.J., Pharmacokinetics of Piroximone (MDL
     19.205) in healthy volunteers. Eur. J. Clin.
     Pharmacol., 31, 239, 1986.

2 - Confidential reports from Merrell Dow : protocol
     099-014, protocols 099-008 and 099-009.

3 - BERG-CANDOLFI, M., BORLAKOGLU, J.T., DULERY, B.,
     JEHL, F., HAEGELE, K.D., Assessment of the
     biotransformation of the cardiotonic agent
     piroximone by high-performance liquid chromatography
     and gas chromatography-mass spectrometry. J.
     Chromatogr 593, 1, 1992.

4 - HOULDSWORTH, C., Merrell Dow Strasbourg research
     team. Actualized analytical method for
     pharmacokinetic studies. Personnal communication.

5 - KEELY, F.J., THEILE, J.D., GARTEIZ, D.A., WEINER,
     D.L., OKERHOLM, R.A., Method of analysis of the new
     cardiotonic agent, MDL 19205, in plasma and urine
     and its application in a dog pharmacokinetic study.
     J. Chromatogr., 278, 379, 1983.

6 - HAEGELE, K.D., HINZE, C., JODER-OHLENBUSCH, A.M.,
     CREMER, G., BORLAK, J., Effects of a standardized
     meal on the pharmacokinetics of the new cardiotonic
     agent Piroximone. Arzneim.-Forsch./Drug Res. 41,
     1225, 1991.

IDENTIFICATION AND DETERMINATION OF GENIPOSIDE, GENIPIN, GARDENOSIDE, AND GENIPOSIDIC ACID FROM HERBS BY HPLC/PHOTODIODE-ARRAY DETECTION

TUNG-HU TSAI[1,2], JEFFREY WESTLY[3],
TZE-FUN LEE[3], AND CHICH-FU CHEN[1,2]
[1]National Research Institute of Chinese Medicine
Taipei, Taiwan
[2]Institute of Pharmacology
National Yang-Ming Medical College
Taipei, Taiwan
[3]Department of Zoology
University of Alberta
Edmonton, Alberta, Canada

ABSTRACT

An improved high-performance liquid chromatographic technique with photodiode-array detection was developed for the identification and determination of the active components geniposide, genipin, gardenoside and geniposidic acid from *Gardenia jasminoides* Ellis and *Gardenia jasminoides* Ellis var. *grandiflora* Nakai. An isocratic system consisting of a reverse-phase phenyl column with a mobile phase of acetonitrile-water-perchloric acid (6:94:0.1, v/v/v, pH 4.0) was used to elute the active ingredients. Variations in extraction methods found that 0.1 M HCl is the best extraction solvent for geniposide and genipin, 0.1 M NaOH for geniposidic acid and water for gardenoside. It was found that water extracts of *Gardenia jasminoides* Ellis contained 56.03 ± 0.62, 1.72 ± 0.01, 2.16 ± 0.04 and 1.79 ± 0.01 mg/g of geniposide, genipin, gardenoside and geniposidic acid respectively. *Gardenia jasminoides* Ellis var. *grandiflora* Nakai, however contained 79.76 ± 0.62, 1.88 ± 0.04, 3.37 ± 0.21 and 6.38 ± 0.13 mg/g.

Correspondence to: C.F. Chen, Institute of Pharmacology, National Yang-Ming Medical College, Taipei 112, Taiwan.

INTRODUCTION

The fruit of both *Gardenia jasminoides* Ellis (Chinese name: Zhi-Zi; ZZ) and *Gardenia jasminoides* Ellis var. *grandiflora* Nakai (Chinese name: Shui-Zhi; SZ) have been used in the traditional Chinese medicine for the treatment of various inflammatory and hepatic-disease [1]. Kimura et al. [2] reported that geniposide, an iridoid glycoside from the fruit of both ZZ and SZ, reduced serum triglyceride, lipid peroxide and phospholipid in rats fed a high sugar diet. The suppressive effect of geniposide on the hepatotoxicity, hepatic DNA binding of aflatoxin B1 in rat [3] and in C3H10T$_{1/2}$ cells [4] have been examined. The crude extracts of ZZ and SZ [5-8] or purified geniposide [9] may also facilitate biliary extraction for treatment of icterus. As both species of *Gardenia jasminoides* are available in the herbal market, it is important to quantitate variations between these herbs. We developed a simple, rapid and sensitive method to identify and to quantify the contents of its major components, geniposide, genipin, gardenoside and geniposidic acid. The variations resulted by different extraction techniques were also examined in the present study.

MATERIAL AND METHOD

Reagent

ZZ and SZ were purchased from a traditional Chinese herbal drug store in Taipei. Authentic gardenoside, geniposide, genipin, and geniposidic acid were obtained from Nacalai Tesque (Kyoto, Japan) and acetonitrile, methanol, n-hexane, ethanol (99.5 %), ammonia solution (32 %) and perchloric acid (70 %) from E. Merck (Darmstadt, Germany).

Apparatus

The HPLC system consisted of an injector (Rheodyne 7125, Cotati, CA, USA) , a Waters Model 990 photodiode-array detector (Milford, MA, USA) and a chromatographic pump (Waters Model 510). Separation was achieved on a Nova-Pak reversed-phase phenyl column (Waters, 150 x 3.9 mm, particle size 5 μm) at room temperature. The mobile phase was acetonitrile-water-perchloric acid (6:94:0.1, v/v/v, pH 4, adjusted by ammonia solution) at a flow rate of 1.0 ml/min. The detection wavelength was 238 nm.

Extraction

ZZ or SZ powder (0.5 g) was boiled for 15 minutes with 50 ml of one of the following extraction solvents: water, methanol, 50 % ethanol, 0.1 M HCl, or 0.1 M NaOH. This procedure was repeated twice and the two filtrates were combined and diluted to a final volume of 100 ml.

Authenticity of samples

The compounds separated by the proposed HPLC method were identified by comparing their retention times and spectra with those of authentic samples of geniposide, genipin, gardenoside and geniposidic acid.

Determination of geniposide, genipin, gardenoside and geniposidic acid

Calibration curves for geniposide, genipin, gardenoside and geniposidic acid in methanol were constructed with various concentration of these compounds (0.1, 0.2, 0.5, 1 and 2 μg). The contents of these components in the crude extract of ZZ and SZ was determined from a regression equation for the area under the curve verses concentration of these four components.
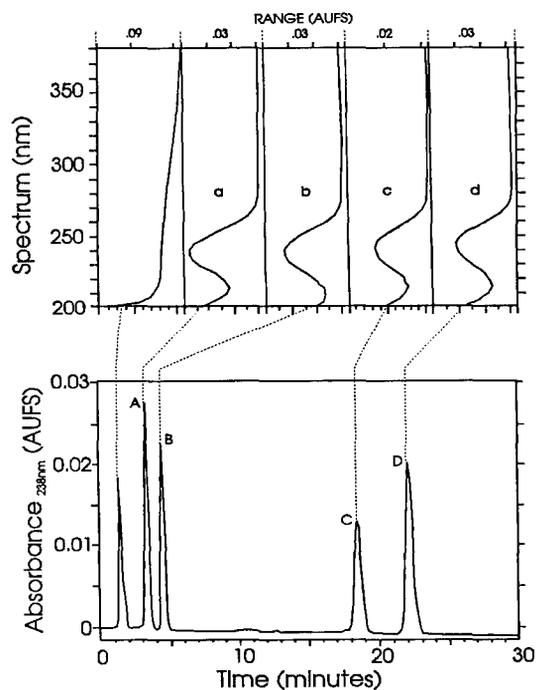
Fig. 1. Chromatogram and UV spectra of authentic compound. A: geniposidic acid; B: gardenoside; C: geniposide; D: genipin.

Statistics

ANOVA with post hoc analysis was used to compare variations between ZZ and SZ and those resulted by different extraction methods.

RESULTS AND DISCUSSION

Fig. 1 shows the chromatogram and UV spectra of authentic geniposide, genipin, gardenoside and geniposidic acid. The peaks corresponding to these four compounds
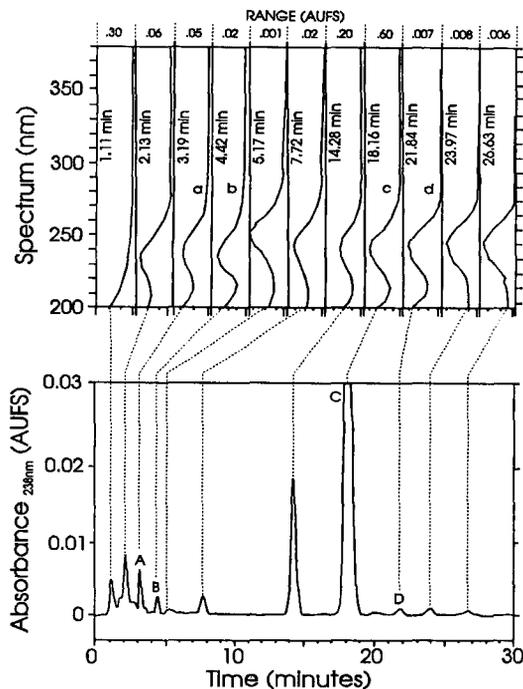
Fig. 2. Chromatogram and UV spectra of water extract of *Gardenia jasminoides* Ellis.
A: geniposidic acid; B:gardenoside; C: geniposide; D: genipin.

were confirmed by the retention times and the UV spectra obtained with photodiode-array

detection. The retention times of geniposide, genipin, gardenoside and geniposidic acid

were found to be 3.5, 4.7, 18.3 and 22.1 min, respectively. The spectra's characteristics

suggested an absorption maxima at 238 nm for geniposide, gardenoside and geniposidic

acid, and 243 nm for genipin in this mobile phase.

The equations of the calibration curve for geniposide, genipin, gardenoside and

geniposidic acid were $y = 0.0232x + 0.0147$ ($r^2 = 0.998$), $y = 0.0158x - 0.0012$ (r^2

$= 0.999$), $y = 0.0143x + 0.0033$ ($r^2 = 0.999$) and $y = 0.0179x + 0.0004$ ($r^2 = 0.999$)

TABLE I

Contents of geniposide, genipin, gardenoside and geniposidic acid in different extracts of *Gardenia jasminoides* Ellis (ZZ) and *Gardenia jasminoides* Ellis var. *grandiflora* Nakai (SZ).

extract solution		geniposide	genipin	gardenoside	geniposidic acid
Water	ZZ	56.03±0.62	1.72±0.01	2.16±0.04	1.79±0.01
	SZ	79.76±1.60[a]	1.88±0.04[a]	3.37±0.21[a]	6.38±0.13[a]
Methanol	ZZ	48.52±0.77	n.d.	n.d.	0.63±0.16
	SZ	76.49±1.90[a]	n.d.	n.d.	3.98±0.40[a]
50% Ethanol	ZZ	54.74±0.98	1.39±0.16	1.22±0.04	0.79±0.01
	SZ	80.01±2.52[a]	2.63±0.07[a]	1.41±0.05[a]	4.11±0.14[a]
0.1 M HCl	ZZ	56.76±1.06	3.64±0.04	0.62±0.03	1.76±0.06
	SZ	82.80±1.88[a]	3.62±0.21[a]	0.91±0.05[a]	8.34±0.27[a]
0.1 M NaOH	ZZ	n.d.	n.d.	n.d.	6.74±0.26
	SZ	n.d.	n.d.	n.d.	9.17±0.45[a]

[a]Significantly different ($p < 0.05$) from ZZ.
Data are expressed as mean±SD (mg/g, n=4).
　　n.d.: not detectable

respectively, where x is the amount of compound analyzed and y is response in peak area. The detection limits for the four compounds, at a signal-to-noise ratio of 4, were 1 ng for geniposide, gardenoside and geniposidic acid, and 3 ng for genipin.

Fig. 2 shows the chromatogram and UV spectra of the water extract of ZZ. The peak corresponding to geniposide, genipin, gardenoside and geniposidic acid were confirmed by both the retention time and the UV spectra.   Table I summarizes the contents of geniposide, genipin, gardenoside and geniposidic acid in ZZ and SZ obtained from the different solvents.   Significant difference was observed between the two

variations of *Gardenia jasminoides* with SZ being consistently higher in content of these components. The method of extraction also gave very significant differences in quantity of component extracted. Highest yield of geniposide and genipin was obtained with 0.1 M HCl extraction, whereas highest yields of geniposideic acid, gardenoside were obtained from 0.1 M NaOH and water extraction, respectively.

In conclusion, the proposed technique should be useful to quantitate the content of quality of either ZZ or SZ.

REFERENCES

1. Juangsu New Medical College, Zhong Yao Da Ci Dian (Dictionary of Chinese Materia Medical), Shanghai Scientific and Technological Publishers, Shanghai, 1985.

2. Y. Kimura, H. Okuda and S. Arichi, Chem. Pharm. Bull., 30: 4444-4447(1982)

3. C.J. Wang, S.W. Wang and J.K. Lin, Cancer Lett., 60: 95-102(1991)

4. T.H. Tseng, C.Y. Chu and C.J. Wang, Cancer Lett., 62: 233-242(1992)

5. T. Miwa, Jap. J. Pharmacol., 2: 102-108(1953)

6. T. Miwa, Jap. J. Pharmacol., 2: 139-143(1953)

7. T. Miwa, Jap. J. Pharmacol., 3: 1-5(1953)

8. T. Miwa, Jap. J. Pharmacol., 4: 69-81(1954)

9. F.T.K. Lau and R.C.K. Pak, Asia Pacific J. Pharmacol. 1: 91-98(1986)

STUDY OF MACROBIOMOLECULE-LIGAND INTERACTIONS BY LIQUID-CHROMATOGRAPHIC SEPARATION METHODS UNDER EQUILIBRIUM AND NONEQUILIBRIUM CONDITIONS

LADISLAV ŠOLTÉS[1] AND BERNARD SÉBILLE

[1]Institute of Experimental Pharmacology
Slovak Academy of Sciences
SQ-84216 Bratislava, Slovak Republic
[2]Laboratory of Physical Chemistry of Biopolymers
University Paris XII
F-94320 Thiais, France

ABSTRACT

The interaction of propranolol with pure human α_1-acid glycoprotein as well as that of bilirubin with complete human serum was studied using high-performance liquid chromatography. Hummel and Dreyer (equilibrium) size exclusion as well as (nonequilibrium) zonal elution chromatographic methods were applied. The binding interaction data were evaluated according to three different approaches introduced by Scatchard, Bjerrum, and by Tobler and Engel.

Procedure schedules designed for the study and evaluation of the reversible (equilibrium) macrobiomolecule-ligand interaction are discussed and critically commented.

INTRODUCTION

Binding interactions of a macrobiomolecule with a ligand resulting in the development of a complex, an associate, represented by e.g. an enzyme-substrate, antigen-antibody, receptor-hormone, have been the subject of studies in several (bio-)chemical, biological, medical, and interdisciplinary branches [1, 2].

The binding parameters of "weakly" interacting components, typical examples of which are protein and drug containing systems, forming a homogenous liquid phase have been most frequently determined by using the method of equilibrium dialysis. The binding characteristics of strongly interacting components in "heterogenous" systems have been predominantly studied by membrane (ultra-)filtration.

This paper presents and comments the results of HPLC studies of two interacting systems, i.e.

- human α_1-acid glycoprotein with propranolol, and

- human serum with bilirubin.

The commentary addresses methodological questions, focusing on the classification of the analytical HPLC methods used, as well as on the procedures applied for the evaluation of the experimental data.

MATERIALS AND DATA ANALYSIS

Ligands and Chemicals

Propranolol racemate (ICI Pharmaceuticals, Macclesfield, Great Britain), bilirubin *p.a.* (Merck, Darmstadt, Germany).

Dithioerytritol 99% (DTE; Aldrich-Europe, Beerse, Belgium). KH_2PO_4, Na_2HPO_4 x 12 H_2O, NaOH, all of *p.a.* purity grade (Merck, Darmstadt). The water used was of Millipore Q quality (Millipore Corporation, Bedford, Ma, U.S.A.).

Proteins and Biological Samples

Human α_1-acid glycoprotein (α_1-AGP; Op.-Nr. 8376, Behring Institute, Germany), human serum albumin (HSA; No. A 1887, Sigma Chemical Co., St. Louis, Mo, U.S.A.).

Nonhemolyzed serum of a healthy adult (AS; 2.9 mg of the total bilirubin per liter) and sera of two newborn infants (IS_1, IS_2; 129 mg/l and 102 mg/l, respectively) were from the Centre Hospitalier Intercommunal de Créteil, France.

Working Solutions

The bilirubin working solution was prepared by dissolving 20 mg of bilirubin in 3 ml of aqueous NaOH (0.1 mol/l). The volume of the solution, to which 30 mg of DTE was added, was supplemented to 10 ml by phosphate buffer solution (0.01 mol/l, pH 7.4).

The blank solution was prepared by the same way, but without bilirubin.

Working Serum Samples

Samples for the HPLC analysis were prepared by mixing the bilirubin working solution and the blank solution with the serum. In the analyzed set of samples, coded/marked from a to m, the dilution factor of the serum was 0.6. (The volume of the injected sample, 10 µl, thus consisted of 6-µl of native serum and of 4-µl of the diluent.)

The concentration of exogenous bilirubin in the diluent for the preparation of a-m samples was: 0, 0.0625, 0.125, 0.1875, 0.25, 0.3125, 0.375, 0.5, 0.625, 0.75, 1, 1.5, and 2 mg/ml.

Scatchard Plot

The binding isotherm of a ligand interaction exclusively with one single class of specific, saturable, binding sites on the macrobiomolecule/protein is usually described by the following equation –

$$B = nkF/(1 + kF) \tag{1},$$

where B is the number of moles of the ligand bound per one mole of the protein, n represents the total number of binding sites, k characterizes the association constant of the given ligand against particular binding sites on the protein, and F is the molar concentration of the free, unbound ligand fraction.

Equation (1) or its manipulated form B/F = nk - kB [3] is frequently used for the graphical evaluation of binding of the drug to the receptor. The linear dependence B/F vs. B, simply called also "Scatchard plot", has the following characteristics [4]:

- the negatively taken direction of the line is equal to the value of the association constant k,

- the intercept on the abscissa equals the value of the total number of binding sites on the protein, n.

Bjerrum Plot

The presentation of the binding data in the form of the so-called "Bjerrum plot" - B vs. logarithm F [2] - proved to be especially advantageous for the assessment of the saturability of the examined binding interaction [4-6]. The B vs. logF dependence has also some characteristic features [4]:

- the S-shaped functional dependence is symmetric with respect to its middle, i.e. to its inflection point,

- the localization of the inflection point in relation to the ordinate (B) represents exactly one half of the total number of binding sites on the protein, i.e. $n_{1/2}$,

- on saturating all binding sites, that is when the concentration of the free ligand fraction is approaching an infinitely high level, the ordinate value (B) of the S-shaped curve reaches the value equal to n.

RESULTS

Figure 1, record c, represents the chromatogram of the given amount of α_1-AGP dissolved in the eluent, i.e. in phosphate buffered propranolol solution. The first, positively oriented, peak belongs to the protein-drug complex. The second peak, with opposite orientation, signalizes the drug deficit in the analyzed sample caused by the binding of propranolol to α_1-AGP.

The amount of the drug fraction bound to the protein was determined by the internal calibration technique [7-11] on the basis of HPLC analysis of the set of samples which are characterized by the constant α_1-AGP concentration but
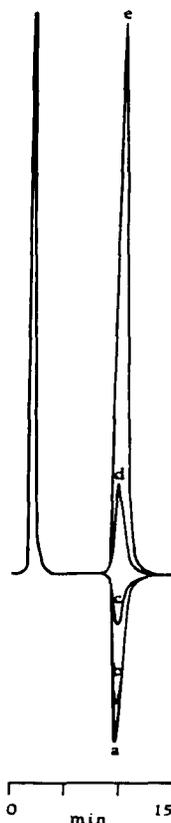
FIGURE 1. Chromatograms of human α_1-AGP (0.6 g/l) dissolved in phosphate buffered solution (0.067 mol/l, pH 7.4) of propranolol, with the drug concentration of 1.0 x 10^{-5} (a), 2.5 x 10^{-5} (b), 5.0 x 10^{-5} (c), 1.0 x 10^{-4} (d), and 2.5 x 10^{-4} (e) mol/l.

Column: 4.7 mm x 15 cm
Column temperature: 37.0 °C
Sorbent: LiChrosorb Diol, 10 μm
Eluent: Phosphate buffered solution (0.067 mol/l, pH 7.4) of propranolol with the concentration of 5.0 x 10^{-5} mol/l
Flow rate: 1 ml/min
Volume of the injected sample: 25 μl
Detector: Spectrophotometer 450; Abs. = 291 nm

by variable propranolol concentrations (figure 1, records
a-e). For example, on using this procedure for the set
propranolol concentration in the mobile phase F = 5.0
x 10^{-5} mol/l the corresponding B value was found to be 1.31
(see figure 2, panel b). On changing the experimental
conditions, i.e. at various drug-concentrations set in the
eluent, for each selected F_i value its corresponding B_i
value was determined.

Figure 2, panels a) and b), shows the binding
interaction of the human α_1-AGP with propranolol as discrete
points plotted in the form of B/F $vs.$ B as well as B $vs.$ F.
As evident from panel a), it is impossible to fit the
experimental points by a straight line, and thus
conclusively, α_1-AGP does not interact with propranolol
exclusively within one single class of specific, saturable,
binding sites.

Figure 3, record a, represents the chromatographic
elution profile of the diluted native serum of a healthy
adult. The HPLC records b-m show the distribution of the
gradually increasing addition of the pigment - exogenous
bilirubin - to the examined AS serum sample. The peak eluted
first represents β-lipoprotein "stained" with the increasing
amount of exogenous pigment; the following peak belongs to
the albumin-bilirubin complex. On the records g-m, the
plateaus indicate the presence of a free/dissociated pigment
fraction in the analyzed samples, most probably due to the
saturation of the capacity of serum albumin and
β-lipoprotein to bind bilirubin.

Figure 4, record a, shows the chromatogram of the
diluted native infant serum (IS_2). Contrary to the healthy
adult serum sample with a similar total bilirubin
concentration (cf. figure 4, record a, and figure 3, record
c or d), the HPLC analysis of the native IS_2 sample
exhibited a significant amount (plateau) of the
free/dissociated endogenous pigment fraction. As evident
from the records b-m (figure 4) the increasing amounts of
exogenous bilirubin added to the IS_2 sample are virtually
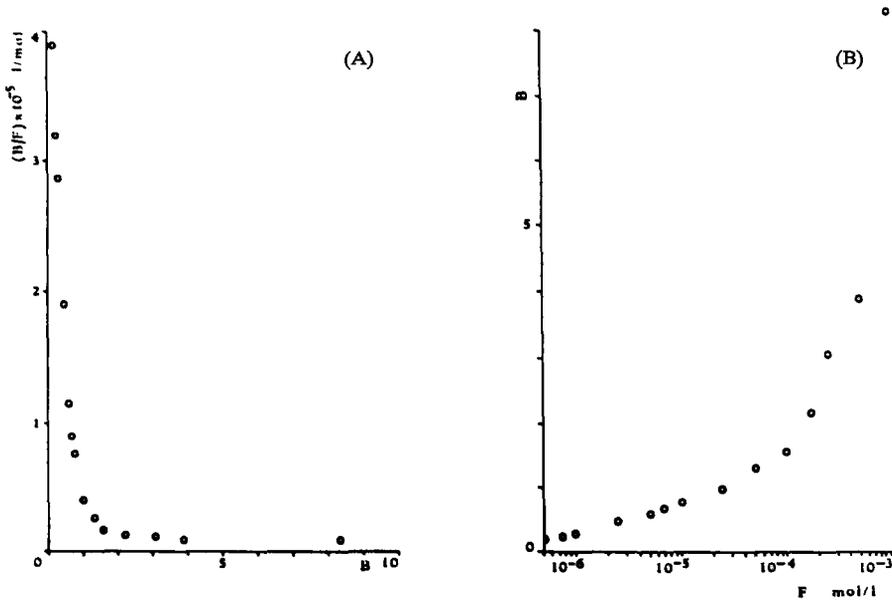
FIGURE   2. Binding   interaction   of   human α_1-AGP   with propranolol.

proportionally distributed between   the bound and free pigment fraction.

DISCUSSION AND COMMENTARY

     The interaction of a macrobiomolecule, a protein (P), with a low-molecular-weight compound, a ligand (L), can be generally expressed by the following chemical equations –

$$P + L \;\; \rightarrow PL \qquad\qquad (2),$$

$$P + L \leftrightarrow P\text{-}L \qquad\qquad (3).$$
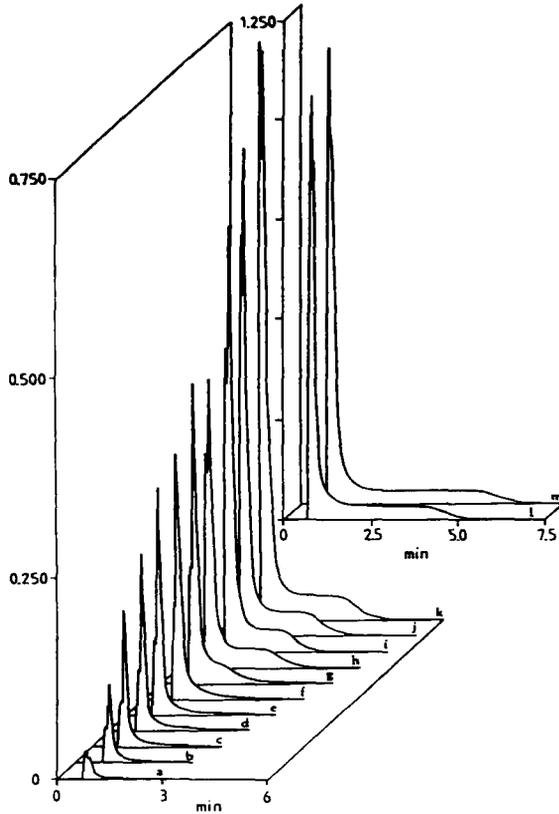
FIGURE 3. Chromatograms of serum of a healthy adult (AS) with the total bilirubin concentration in individual samples: 1.74 (a), 26.74 (b), 51.74 (c), 76.74 (d), 101.74 (e), 126.74 (f), 151.74 (g), 201.74 (h), 251.74 (i), 301.74 (j), 401.74 (k), 601.74 (l), and 801.74 (m) mg/l.

Eluent: Phosphate buffered solution (0.067 mol/l, pH 7.4) with the concentration of 80 mg HSA/l and with the addition of the bilirubin working solution = 1000:0.05
Column: 4.7 mm x 15 cm
Sorbent: LiChrosorb Diol, 5μm
Flow rate: 1 ml/min
Detector: Filter photometer 440; Abs. > 436 nm
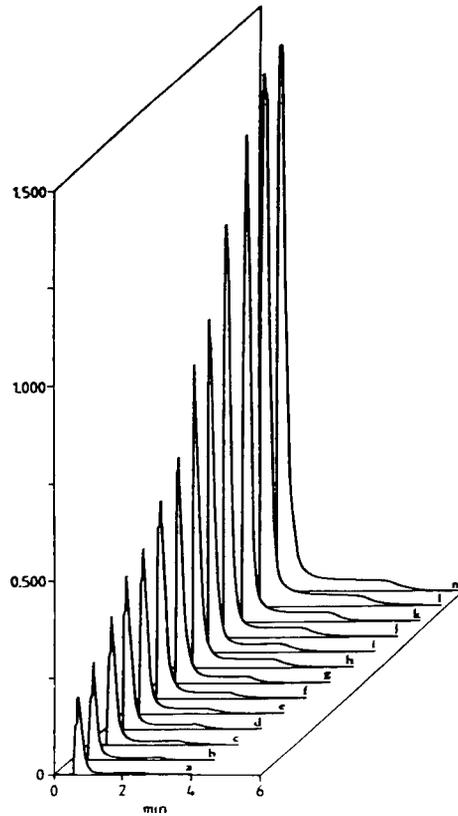
FIGURE 4.   HPLC elution profiles of   infant serum (IS$_2$) with
the total   bilirubin level in individual   samples: 61.2 (a),
86.2 (b), 111.2 (c), 136.2   (d), 161.2 (e), 186.2 (f), 211.2
(g), 261.2 (h), 311.2 (i),   361.2 (j), 461.2 (k), 661.2 (l),
and 861.2 (m) mg/l.

Eluent: Phosphate buffered   solution (0.067   mol/l, pH 7.4)
with the concentration of 2 ml AS/l and with the addition of
the bilirubin working solution = 1000:0.05

(Column, sorbent, flow rate, detector - see figure 3.)

Equation (2) characterizes an irreversible reaction where a qualitatively new substance - the product PL - is formed from the reactants P and L. In living organisms such a (bio-)chemical reaction, diminishing the endogenous protein and simultaneously generating a new xeno-macrobiomolecule PL, characterizes usually an adverse pathophysiological or even pathological process.

Finding of a PL-type substance, e.g. in human blood, can sometimes directly serve as an indicator of a certain disease. Besides sugar in urine, the diagnosis of *diabetes mellitus* is indicated also by glycosylated albumin in blood plasma.

In *in vitro* experiments, the stechiometry of the reaction expressed by equation (2) is studied usually on using a radioisotope labeled ligand (L^*). The ternary mixture of the reactants P and L^* and the product PL^* is separated only into two fractions a low-molecular of the nonconverted ligand L^* and a macromolecular one which is a binary mixture of the protein P and the substance PL^*.

For quick and efficient separation of two fractions differing in their molecular weights the HPLC method working under size exclusion chromatographic mode is being primarily applied. The question of the quantification of the amount of the nonconverted ligand and of the generated product is, thanks to the application of the labeled reactant L^*, simplified to measuring the radioactivity of the two fractions, i.e. PL^* + P, and L* [12].

Unlike the chemical reaction described by equation (2), which in the living body represents adverse processes, the reversible interaction expressed by equation (3) involves several processes which are essential for physiological functioning of the organism. Many such reversible interactions of effector molecules (hormone, substrate, low- or high-molecular-weight antigen) with corresponding macrobiomolecules (receptor, enzyme, antibody) do not only constitute the basis of biochemical regulation, signalization and defense of particular animal cells, but

also the basis of (bio-)chemical "communication" between cells in the whole body.

However, the quantitative stechiometric analysis of the reaction described by equation (3) is a very complicated task. This difficulty results from the demand to determine the instantaneous molar concentrations (molar activities [P], [L], and [P-L]) of each component of the ternary mixture without deranging the equilibrium described by equation (3) and from the fact that the "product" P-L is a complex, an associate, which actually does not exist in the form of a pure, isolated chemical item.

The Method of Hummel and Dreyer

In 1962 Hummel and Dreyer were first to describe [7] a liquid chromatographic separation method which has been applied in studying reversible interactions between a macrobiomolecule and a ligand [10, 13-18]. The characteristic feature of the technique developed by Hummel and Dreyer is the equilibration of the chromatographic column packing with the mobile phase which contains a given ligand (drug) concentration. (The most frequently used column fillings are sorbents which work in the mode of size exclusion chromatography or in the separation mode based on ion-exchange effects. The major requirement for the applicability of a particular sorbent is its "inertness" against the injected sample containing a protein). The injected, analyzed sample is a certain amount of the macrobiomolecule dissolved in the eluent.

The injection of such a sample usually yields two peaks in the resulting chromatographic record (see figure 1, record c). The first (positive) peak belongs to the protein-drug complex. Its retention volume usually equals the void volume of the chromatographic column used. The second, negatively oriented peak, which is detected at the retention volume of the drug, manifests the ligand deficit

in the analyzed sample caused by the association of a particular drug fraction with the given amount of the protein.

The amount of the protein-bound drug fraction is determined by the internal (see figure 1) or external calibration technique [19]. The purpose of the use of either calibration technique is to find such a concentration of the ligand which just compensates the drug deficit in the analyzed sample, or to determine that ligand excess which eliminates the appearance of the second peak on the chromatographic record.

The rationale of the Hummel and Dreyer method is that the rate of the stabilization of the composition of the protein-ligand complex injected into the eluent stream containing the given concentration of the free drug is much higher than the speed of the chromatographic process itself [18]. Since in practice this condition is fulfilled usually *a priori*, the Hummel and Dreyer method is generally declared to be an equilibrium liquid chromatographic separation method for studying reversible macrobiomolecule-ligand interactions.

However, despite the fact that the number of the ligand moles bound by one mole of the protein (B) depends exclusively on the drug concentration set in the eluent (F), the gradual dilution of the protein-ligand complex, which naturally occurs during the chromatographic process, can significantly influence the macrobiomolecule-drug interaction equilibrium. As a result of this process, the determined binding parameters are only apparent/"pseudoequilibrated" [20].

Another critically not yet evaluated characteristic feature of any liquid chromatographic study of a reversibly interacting system, including the method introduced by Hummel and Dreyer, is a pressure drop along the chromatographic column which may result in a permanent change of the protein-drug complex composition when it is transported through the column body.

Binding Data Analysis

The reversible interaction of a ligand with an acceptor macrobiomolecule is generally described by the following equation [3, 21] -

$$B = \sum_{i=1}^{N} n_i k_i F/(1 + k_i F) \tag{4},$$

where n_i represents the number and k_i the affinity constant of the i-class of binding sites for which the following limits are simultaneously valid:

- all binding sites on the macrobiomolecule exist independently of the presence or absence of the ligand in the system and, due to the interaction of the two components, the binding sites do neither arise nor disappear,

- each binding site on the macrobiomolecule interacts with the ligand independently.

Yet the validity of the above mentioned limiting conditions, and thus the aplicability of the dependence (4) called also Scatchard equation, is equivocal since neither the plasmatic protein nor the receptor protein exist in the system in a "frozen" conformation carrying pre-existing classes of binding sites characterized by fixed affinities for the given ligand [22]. Moreover, the evaluated constants have only a pseudovalue [2, 23] because the solution of equation (4) is not an unambiguous, objective, set of values of n_i and k_i pairs.

Therefore, on comparing binding data from various laboratories it is recommended to consider the shape and the position of the binding isotherms and not the numeric values of the binding constants [23].

The Tobler and Engel Plot

In 1983 Tobler and Engel published an original procedure of computer analysis of equilibrium binding

interactions, the result of which is the so-called Affinity
spectrum [24]. This spectrum (plot) shows the number of
binding sites *vs.* corresponding dissociation constants
$(1/k_i)$.

The input data are represented exclusively by the set
of values F_i and B_i. The evaluation based on the linear
programing principle results in pairs n_i and $1/k_i$, the
values of which can be read from the given plot both
visually and in the form of numeric data [24].

The affinity spectrum (Tobler and Engel plot) of the
system containing α_1-AGP and propranolol (see figure 5)
indicates as the most adequate description of the binding
data the isotherm in the following form -

$$B = \sum_{i=1}^{N} n_i k_i F/(1 + k_i F) + n'k'F \qquad (5)$$

whith the number of mutually independent classes of specific
binding sites $N = 2$, while the product n'k'F characterizes
the simultaneous manifestation of nonspecific (unsaturable)
binding. But this result of binding data computer analysis
of the system comprising human α_1-AGP and propranolol
racemate lends itself also to the interpretation that the
generated affinity spectrum allowed to recognize the
simultaneous manifestation of two different reversible
interactions between the given protein and individual
propranolol enantiomers. (The system containing the chiral
macrobiomolecule, α_1-AGP, and the drug which is the mixture
of two enantiomers has in fact three components.)

Binding Interaction of Human α_1-AGP with (+)- and (-)-Propranolol

Despite the fact that the (-)-propranolol enantiomer is
pharmacologically up to 100-times more effective than its
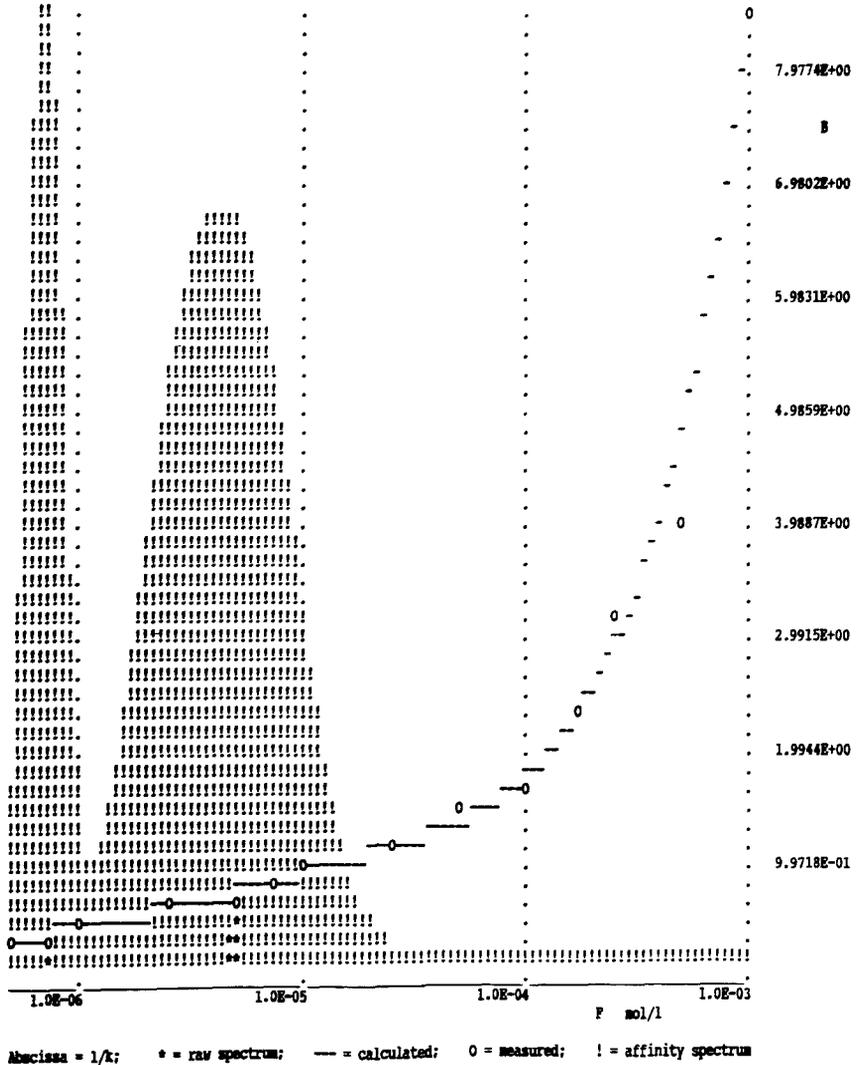
FIGURE 5.   Tobler and Engel plot (affinity spectrum) of the binding interaction between human α_1-AGP and propranolol.

(+)-isomer, in therapeutic practice this β-adrenoceptor blocking drug is administered in the form of a racemic mixture of both its optical antipodes. Thus propranolol racemate was used in the majority of determinations concerning the interaction of this β-adrenolytic even with individual proteins [10, 25-30].

So far only few laboratories have been studying the binding interactions of individual propranolol enantiomers, using either complete blood plasma from various animals (human, dog, rat) or also some isolated and purified plasma proteins (α_1-AGP, HSA) [31-35]. However, due to the lack of any exact knowledge about the stereospecifity of the reversible interaction of macrobiomolecules with individual propranolol enantiomers at molecular level, the results published so far can be classified as relatively highly inconsistent. The value and relevance of the stated binding parameters is also doubtful, both due to the use of not standardly pure proteins (e.g. α_1-AGP) and mainly due to the evaluation of experimental data by using non-adequate mathematical description of the corresponding binding isotherm.

Reversible interactions in both systems containing standardized human α_1-AGP with the particular (+)- or (-)-propranolol enantiomer have been characterized only in the senior author's Laboratory. For these measurements the HPLC method according to Hummel and Dreyer was used [36, 37].

By using this method and by combining the processes of the experimental data analysis according to Scatchard, Bjerrum, as well as to Tobler and Engel, the determined binding interaction between α_1-AGP and (-)-propranolol was found to be saturable, with the parameters n = 0.81 and k = 2.73 x 10^5 1/mol. The binding isotherm of the system α_1-AGP and (+)-propranolol was adequately described by the interaction parameters n = 0.38, k = 3.39 x 10^6 1/mol, and by the value of n'k'= 1.39 x 10^4 1/mol.

The measurement of circular dichroism in both systems, i.e. human α_1-AGP with (-)- or (+)-propranolol enantiomer [36], confirmed the adequacy of evaluating the interaction between the protein and each individual isomer with the use of different binding isotherms expressed by equation (1), or equation (5) with the value of N = 1.

Retention Analysis with Zonal Elution Chromatography

Qualitative as well as quantitative evaluation of the interaction   between   a   macrobiomolecule   and a low-molecular-weight ligand can be done also on the basis of measuring the kinetics of the protein-ligand complex dissociation by the method of zonal elution chromatography [11, 38, 39]. On using this technique, a certain amount of the sample - the solution of the protein plus ligand - is injected into the chromatographic column eluted with the solvent of the analyzed sample, most frequently phosphate buffer. For the separation of the sample components the mode of size exclusion, ion-exchanging, affinity chromatography, *etc*. is used.

In general, the protein-ligand complex (together with the protein surplus) is eluted from the column first followed by another peak belonging to the free, non-associated ligand. However, it is also necessary to emphasize that in zonal elution chromatography the protein-ligand complex, while transported through the column, continually dissociates both because of its constant mixing and gradual dilution by the eluent and because of the ligand retardation by the stationary phase. (The disintegration process of 10 % of the complex characterized by the association constant value k = 10^{10} 1/mol lasts for about 1000 seconds; when k = 10^9 1/mol this process lasts only 100 seconds, etc.)

Besides the above mentioned facts, however, the method of zonal elution chromatography   is primarily used in

studying "solid" complexes, typical examples of which are antigen-antibody, enzyme-substrate, etc. The selection of zonal elution chromatography is favored mainly by the fact that this technique works effectively also with minute amounts of material usually available in immunology, enzymology, and in many other scientific fields and medical branches, as e.g. in pediatrics.

Interaction of Human Serum with Bilirubin

The serum capacity to bind bilirubin has to be determined very often in pediatrics in assessing the course of neonatal jaundice. One of the methods for determining the reserve bilirubin binding capacity of the infant serum is based on the sample "filtration" through the Sephadex G-25 minicolumn bed. The bilirubin fraction firmly bound to the proteins passes through the column while the fraction of "free" (loosely bound) pigment remains adsorbed onto the packing material. This free/dissociated bilirubin fraction is eluted from the minicolumn with the help of an alkaline solution, in the next step the pigment is extracted into the chloroform and finally quantified spectrophotometrically.

The serum reserve bilirubin binding capacity is thus determined by titrating the sample with exogenous bilirubin and by the subsequent determination/demonstration of a free pigment portion. By using a similar filtration technique through Sephadex G-100, Cooke and Roberts found [40] that the exogenous bilirubin added to the adult serum associated with two proteins - with albumin and β-lipoprotein.

On applying this method of filtrating a bilirubin stained sample through the gel bed the following important phenomena should however be taken into account:

- The molecular weight of the β-lipoprotein ($\approx 2 \times 10^6$ Da) is significantly higher than that of albumin (66210 Da [41]). This is why at gel filtration of stained as well as

not-stained proteins β-lipoprotein is first eluted from the
sorbent, followed by albumin.

- The Sephadex type adsorbent has a relatively high affinity
to   bilirubin.   (After   application   of   the   stained
β-lipoprotein, the Sephadex remains colored as the result of
both the process of complex dissociation and of adsorption
of the dissociated bilirubin portion by the sorbent [40].)

- Because of a certain albumin reserve capacity to bind
bilirubin, this   protein,   eluted   after the β-lipoprotein,
desorbs/extracts   a   part   of   the   pigment adsorbed on the
sorbent.

     In   contrast   to   the   above   mentioned   gel filtration
technique, the HPLC method described herein for assessing
the reversible bilirubin binding to human serum proteins
exploits simultaneously the mechanism of the size exclusion
and (ad-)sorption chromatographic separation. Under the
conditions of the presented method the dissociation of the
protein-bilirubin complex was suppressed by addition of
bilirubin to the mobile phase. The solubility of the pigment
in the phosphate buffer solution used (0.067 mol/l, pH 7.4)
was warranted by the "carrier" - HSA [42, 43] or the adult
serum [43].

     On using the presented HPLC method, the fractions of
bilirubin   associated   with   albumin   as   well   as   with
β-lipoprotein,   and   also   the   free   –   loosely
bound/dissociated - pigment portion (see figures 3 and 4)
were determined in serum samples of an adult human and of
newborn infants.

     While in the native IS_1 serum sample the amount of the
free (dissociated) bilirubin fraction represented only 11.5
%, the amount of this fraction in the IS_2 sample was up to
17.3 % (figure 4, record a). The titration of IS_1 and IS_2
sera with exogenous bilirubin demonstrated that in the IS_1
sample the binding capacity of the serum albumin was
significantly higher than that of β-lipoprotein, while in
the IS_2 sample the pigment was associated with
β-lipoprotein and with albumin to a comparable extent [43].

ADDENDUM

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Besides the interaction of human α_1-AGP with (+)- and
(-)-propranolol [36] also reversible binding of the basic
drug propafenone and its (R)- and (S)-enantiomers with
α_1-AGP [44], as well as that of the acidic drug pirprofen
including its (+)- and (-)-optical antipodes with HSA [45]
was studied in the senior author's Laboratory. On the basis
of the acquired experimental experience, it is possible to
suggest the following general design of a study procedure
and   the   evaluation   of   data   on   interactions   between
a macrobiomolecule, protein, and a drug as well as its
isomers/enantiomers –

– measurement of the interaction of the drug (racemate) with
the given protein in an as wide as possible range of values
of the [L]/[P] ratio using the HPLC method (the Hummel and
Dreyer technique),

– measurement of the interaction of the given protein with
individual   drug   enantiomers   by   the   above   mentioned
technique,

– mutual comparison of binding isotherms (Scatchard as well
as Bjerrum plots) of all systems measured,

– processing of the results of interaction measurement of
the given protein with individual drug enantiomers by using
the computer program "AFFINITY SPECTRA" [24],

– comparison of the affinity spectrum of the given system
with characteristics yielded by the Scatchard and/or Bjerrum
plot,

– additional determination of the binding interaction nature
of the studied system containing the corresponding drug
enantiomer by using the method of circular dichroism,
and/or nuclear magnetic resonance [45],

– suggestion of the most plausibly valid binding isotherm of
the system studied,

- mathematico-statistical processing of the experimental
data by using the method of non-linear regression only if
equation (1), or equation (5) with the value of $N = 1$ can be
applied.

CONCLUSION

The study of drug interaction with plasma proteins is
an indispensable phase in the development of new drugs and
in their introduction into clinical practice.

Since the molecules of almost all synthetic drugs have
one or more chiral centers [46, 47] the elaborated
experimental procedure of studying and evaluating the
reversible interaction between a given protein and a drug as
well as its isomers can be classified as an important
methodological contribution.

The HPLC method of the determination of the free and
the protein bound bilirubin fractions in an infant serum
sample represents an efficient diagnostic tool. The
endogenous and exogenous bilirubin distribution between
albumin and β-lipoprotein can be completely determined by
examining even a minute sample volume.

ACKNOWLEDGEMENT

REFERENCES

1. I. M. Klotz, 'Protein Function', T. E. Creghton, ed., p.
   25.

2. R. Brodersen, B. Honoré, A. O. Pedersen, I. M. Klotz, Trends Pharmacol. Sci., <u>9</u>: 252 (1988).

3. G. Scatchard, Ann. N. Y. Acad. Sci. <u>51</u>: 660 (1949).

4. L. Šoltés, B. Sébille, A. Fügedi, Bio-Sciences, <u>8</u>: 13 (1989).

5. I. M. Klotz, Science, <u>217</u>: 1247 (1982).

6. I. M. Klotz, Science, <u>220</u>: 981 (1983).

7. J. P. Hummel, W. J. Dreyer, Biochim. Biophys. Acta, <u>63</u>: 530 (1962).

8. B. Sébille, N. Thuaud, J.-P. Tillement, J. Chromatogr., <u>167</u>: 159 (1978).

9. B. Sébille, N. Thuaud, J.-P. Tillement, in <u>Biological/Biomedical Applications of Liquid Chromatography IV,</u> Vol. 20, G. L. Hawk, ed., Marcel Dekker, Inc., New York and Basel, 1982, p. 31.

10. F. Brée, J.-P. Tillement, B. Sébille, J. Chromatogr., <u>375</u>: 416 (1986).

11. B. Sébille, 'HPLC of Biological Macromolecules', K. M. Gooding, Regnier, F. E., eds., Marcel Dekker, Inc., New York and Basel, 1990, p. 585.

12. P. Chabreček, L. Šoltés, Z. Kállay, M. Guttmann, CS.-Patent Pending, 1990, PV 3292-90.

13. O. Pepin, N. Thuaud, B. Sébille, L. Edouard, N. Lemort, J.-L. Beaumont, Chromatographia, <u>16</u>: 267 (1982).

14. S. F. Sun, S. W. Kuo, R. A. Nash, J. Chromatogr., <u>288</u>: 377 (1984).

15. B. Sébille, N. Thuaud, J. Piquion, N. Behar, J. Chromatogr., <u>409</u>: 61 (1987).

16. G. Berger, G. Girault, J.-M. Galmiche, J. Liq. Chromatogr., <u>12</u>: 535 (1989).

17. J. Mohler, A. Meulemans, M. Vulpillat, J. Chromatogr., <u>528</u>: 415 (1990).

18. T. C. Pinkerton, K. A. Koeplinger, Anal. Chem., <u>62</u>: 2114 (1990).

19. S. F. Sun, F. Wong, Chromatographia, <u>20</u>: 495 (1985).

20. D. L. Parsons, J. Chromatogr., <u>193</u>: 520 (1980).

21. I. M. Klotz, Arch. Biochem., <u>9</u>: 109 (1946).

22. K. Zierler Trends Biochem. Sci., <u>14</u>: 314 (1989).

23. B. Honoré, Pharmacol. Toxicol., <u>66</u>: Suppl. II, (1990).

24. H. J. Tobler, G. Engel, Naunyn-Schmiedeberg's Arch. Pharmacol., <u>322</u>: 183 (1983).

25. S. Glasson, R. Zini, P. d'Athis, J.-P. Tillement, J. R. Boissier, Molec. Pharmacol., <u>17</u>: 187 (1980).

26. W. E. Müller, A. E. Stillbauer, Naunyn-Schmiedeberg's Arch. Pharmacol., 322: 170 (1983).

27. A. K. L. Wong, J. C. Hsia, Can. J. Biochem. Cell Biol., 61: 1114 (1983).

28. F. M. Belpaire, R. A. Braeckman, M. G. Bogaert, Biochem. Pharmacol., 33: 2065 (1984).

29. L. Šoltés, F. Brée, B. Sébille, J.-P. Tillement, M. Ďurišová, T. Trnovec, Biochem. Pharmacol., 34: 4331 (1985).

30. L. Šoltés, B. Sébille, F. Brée, J.-P. Tillement, T. Trnovec, M. Ďurišová, J. Pharmacol. (Paris), 17: 208 (1986).

31. S. A. Bai, U. K. Walle, M. J. Wilson, T. Walle, Drug. Metab. Dispos., 11: 394 (1983).

32. U. K. Walle, T. Walle, S. A. Bai, L. S. Olanoff, Clin. Pharmacol. Ther., 34: 718 (1983).

33. F. Albani, R. Riva, M. Contin, A. Baruzzi, Br. J. Clin. Pharmacol., 18: 244 (1984).

34. H. Takahashi, H. Ogata, Biochem. Pharmacol., 39: 1495 (1990).

35. H. Takahashi, H. Ogata, S. Kanno, H. Takeuchi, J. Pharmacol. Exptl. Ther., 252: 272 (1990).

36. J. Oravcová, S. Bystrický, T. Trnovec, Biochem. Pharmacol., 38: 2575 (1989).

37. J. Oravcová-Kosková, L. Šoltés, A. Fügedi, T. Trnovec, Čs. Fysiol., 38: 166 (1989).

38. B. Sébille, Fundam. Clin. Pharmacol., 4: Suppl. 2, 151s (1990).

39. B. Sébille, R. Zini, C.-V. Madjar, N. Thuaud, J.-P. Tillement, J. Chromatogr., 531: 51 (1990).

40. J. R. Cooke, L. B. Roberts, Clin. Chim. Acta, 26: 425 (1969).

41. C. E. Ahlfors, Anal. Biochem., 110: 295 (1981).

42. L. Šoltés, B. Sébille, J.-P. Tillement, D. Berek, J. Clin. Chem. Clin. Biochem., 27: 935 (1989).

43. L. Šoltés, B. Sébille, A. Fügedi, Clin. Chim. Acta, 201: 35 (1991).

44. J. Oravcová, W. Lindner, P. Szalay, Ľ. Boháčik, T. Trnovec, Chirality, 3: 30 (1991).

45. J. Oravcová, V. Mlynárik, S. Bystrický, L. Šoltés, P. Szalay, Ľ. Boháčik, T. Trnovec, Chirality, 3: 412 (1991).

46. E. J. Ariëns, Med. Res. Rev., 6: 451 (1986).

47. E. Mutschler, Y. Gietl, D. Krauss, E. Martin, G. Pflugmann, H. Weber, H. Spahn, in <u>Chirality and Biological Activity</u>, Alan R. Liss, Inc., 1990, p. 199.

TARTARIC ACID IN WINES MAY BE USEFUL FOR PREVENTING RENAL CALCULI: RAPID DETERMINATION BY HPLC

**L. RADIN, C. PRONZATO,
L. CASARETO, AND L. CALEGARI**
*Institute of Microbiology
University of Genoa
viale Benedetto XV, 10
16132 Genoa, Italy*

ABSTRACT

HPLC has been applied to the analysis of wine samples for the rapid determination of tartaric acid. Separation of carboxylic acids was performed on a LiChrosorb RP-18 column using a mixture of water, methanol and 0.05 M phosphoric acid (69:1:30) as mobile phase. Column eluates were monitored by UV absorbance at 210 nm. Tartaric, malic, lactic, acetic and tannic acids were revealed in the HPLC profile. Tartaric acid eluted as a well-resolved peak at 3.3 min. Mean recovery of known amounts of added tartrate ranges between 94 and 106%. The concentrations of tartaric acid in different European wines have been determined. The presence of tartaric acid can be related to the chemistry of nephrolithiasis: a high amount of this acid in the diet may be useful in preventing the recurrence of calcium oxalate stones in urine. The results of HPLC analyses appear to be interesting for the choice of a wine suitable for the diet of patients suffering from renal calculi.

INTRODUCTION

Wines contain numerous compounds which belong to different classes: alcohols, carboxylic acids, aldheydes and esters. The organoleptic properties of the various types of wines are affected by the carboxylic acid composition. The quality control of wines requires precise determinations of individual carboxylic acids during fermentation and aging.

The main acidic components in wine are tartaric, malic and citric acids (1). Tartaric acid is the major acid which regulates wine acidity. Tartaric acid undergoes degradation by lactic bacteria (such as <u>Lactobacillus brevis</u>) to lactic and acetic acids, with a concomitant increase in volatile acidity.

Most of ingested tartrate is metabolized to bicarbonate by various bacterial species in the colon (2), while only 20% of the dietary tartrate is excreted unchanged in urine (3). Tartrate is a strong chelating agent and may inhibit crystallization of calcium oxalate in whole urine (4). It was pointed out that the alkalinization of urine caused by tartrate feeding might be useful in preventing the recurrence of calcium oxalate stones (5).

Clinical results demonstrated the dependence of tartrate excretion on the composition of diet, as urinary excretion of tartrate was significantly less in subjects on a vegetarian diet than in subjects on mixed Mediterranean diets (3). Other authors reported that the incidence of renal calculi was strikingly low in South India, where one regular constituent of the diet is tamarind, which is very rich in potassium bitartrate and tartaric acid (5). One of

the major exogenous sources of tartrate is wine: due to wine intake, the amount of tartrate excreted can overcome the value of 1 mmol/24 hours (3).

The conventional methods used for the determination of tartrate, such as colorimetry and gas-chromatography, are all quite complex, time consuming and present several drawbacks (6-8). HPLC has been applied to the analysis of carboxylic acids. The methods commonly used have included ion-exchange and ion-exclusion separation (9,10), solvophobic chromatography, ion-pair chromatography and reversed-phase separation of derivatized products. The derivatives most frequently examined have been differently substituted phenacyl, naphthacyl, p-nitrophenyl and p-nitrobenzyl esters (11-13).

The aim of this study was to apply HPLC to the rapid determination of tartaric acid in wines. The amounts of tartaric acid in different European wines have been calculated in order to reveal which wines represent the best defence against recurrent nephrolithiasis.

MATERIALS

Chemicals.

Standard solutions of the investigated acids (tartaric, malic, lactic, acetic, citric, succinic, and tannic) were prepared from analytical-reagent grade chemicals (Merck, Darmstadt, Germany) by dissolving known amounts of compound in distilled water.

The solvents used for the HPLC analyses were of HPLC grade and water was deionized using a Milli-Q system (Millipore, Bedford, MA, U.S.A.). The eluents were filtered through 0.45-µm membrane filters (Millipore) prior to use.

METHODS

HPLC analysis.

Samples of 20 µL of wine filtered through 0.22 µm filters (Bio-Rad Laboratories, Richmond, CA, U.S.A.) were injected into the chromatograph. Analyses were carried out using a Merck-Hitachi liquid chromatograph (Tokyo, Japan), equipped with a model 7125 Rheodyne injector (Cotati, CA, U.S.A.), with a spectrophotometer, and with a model 4290 Varian integrator (Walnut Creek, CA, U.S.A.). Separation of carboxylic acids was performed on a LiChrosorb RP-18 column (25 cm x 4.6 mm) purchased from Merck (Darmstadt, Germany). using a mixture of water – methanol – 0.05 M phosphoric acid (69:1:30) as mobile phase, at a flow-rate of 0.8 mL/min. All chromatographic separations were performed at 22 ± 1°C. Column eluates were monitored by UV absorbance at 210 nm.

The standard solutions of tartaric acid used for calibration were prepared by dissolving the required weight of compound in distilled water. A calibration graph was obtained by analyzing aliquots of working standard solutions containing scalar amounts of tartrate from 0.3 to 3.0 g/L.

The detection limit for tartaric acid was estimated in aqueous samples by injecting successively lower concentrations until a signal-to-noise ratio of 3:1 was obtained.

The recoveries of tartaric acid were determined by adding known amounts of tartrate to six wine samples and by comparing the found and calculated amounts of tartrate after spiking.

The precision of the method was evaluated by calculating within-run and between-run coefficients of variation at four different concentrations of tartrate.

RESULTS

A mixture of major acids (tartaric, malic, lactic, acetic, and tannic) present in wines was used as a standard solution for optimizing the chromatographic conditions. The elution behaviour of each acid was investigated at various pH values of eluent. The retention times decreased with increasing pH. Lactic and acetic acids were not completely separated at pH > 3. Separation of each acid was examined at different concentrations of methanol in the mobile phase. The best resolution of all the acids was obtained by using a methanol concentration of 1%. Each acid was identified by comparison with the retention times of single pure
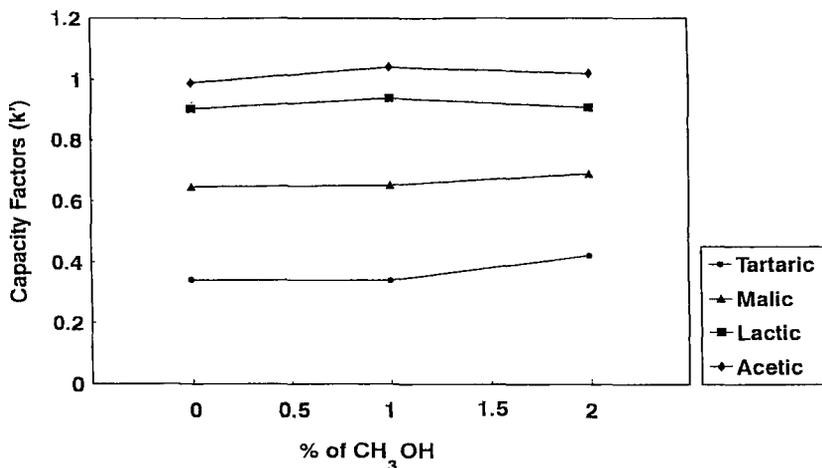
FIGURE 1.
Effect of methanol concentration on the capacity factor of carboxylic acids.
Column: LiChrosorb RP-18 (250 x 4 mm i.d.); mobile phase: water - 0.05 M phosphoric acid (70:30); flow-rate: 0.8 mL/min; temperature: 22°C.

compounds. Relationships between capacity factors and methanol concentrations, for carboxylic acids, are presented in Fig. 1. Results of these experiments have shown that a mobile phase containing 1% methanol at pH 2.5 was suitable for a correct separation of tartaric acid.

Other acids, such as succinic and citric, included in the standard mixture were not completely separated also varying pH and methanol concentration, however, their presence in wines is not important for organoleptic properties.

HPLC profile of a standard mixture of carboxylic acids is shown in Fig. 2. The total analysis time was 18 min,
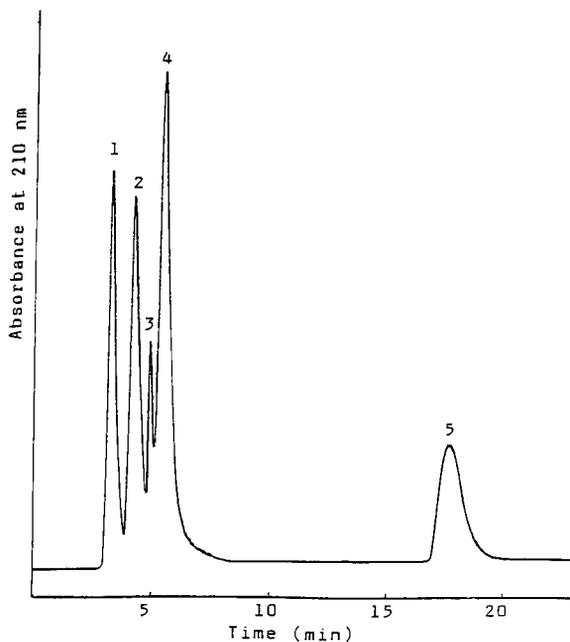
FIGURE 2.
HPLC profile of a standard mixture of carboxylic acids.
Column: LiChrosorb RP-18 (250 x 4 mm i.d.); mobile phase:
water - methanol - 0.05 M phosphoric acid (69:1:30);
flow-rate: 0.8 mL/min; temperature: 22°C. Peaks: 1 =
tartaric acid; 2 = malic acid; 3 = lactic acid; 4 = acetic
acid; 5 = tannic acid.

while the elution of tartaric acid was very quickly at 3.3
min before the other acids.

HPLC patterns of a sample of Rossese di Finale Ligure
(Italy) and Beaujolais (France) wines are shown in Figs. 3
and 4, respectively.
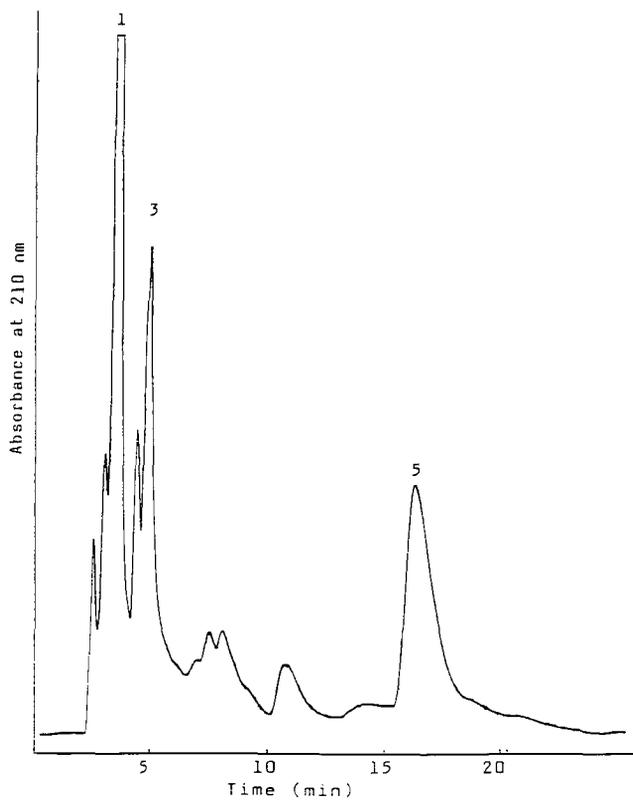
FIGURE 3.
HPLC profile of a sample of Rossese di Finale Ligure wine.
Chromatographic conditions as in Fig. 2. Peaks: 1 = tartaric
acid; 3 = lactic acid; 5 = tannic acid.
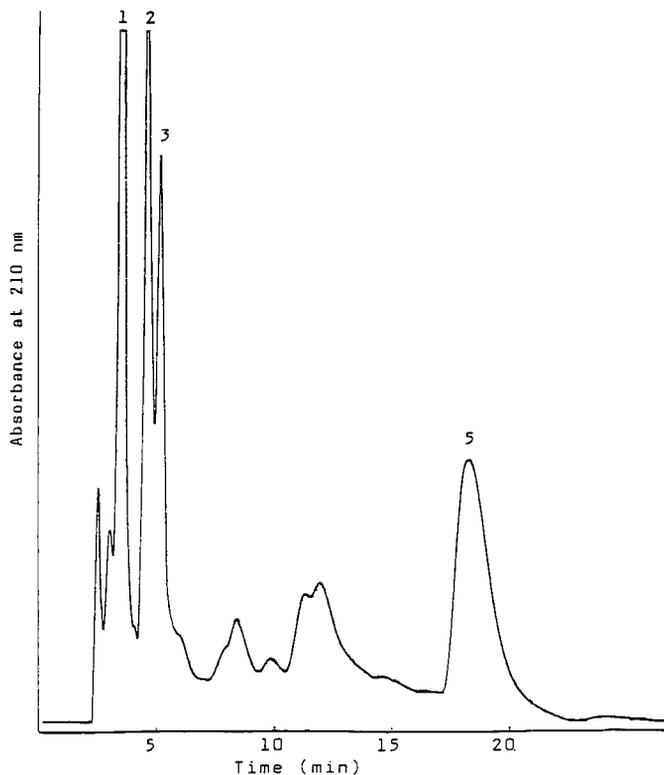
FIGURE 4.
HPLC profile of a sample of Beaujolais wine.
Chromatographic conditions as in Fig. 2. Peaks: 1 = tartaric
acid; 2 = malic acid; 3 = lactic acid; 5 = tannic acid.

Quantitative determination.

    The calibration graph relating tartrate peak area to
concentration was produced by sequential dilution of a stock
solution in the range 0.3 — 3.0 g/L. The response of the
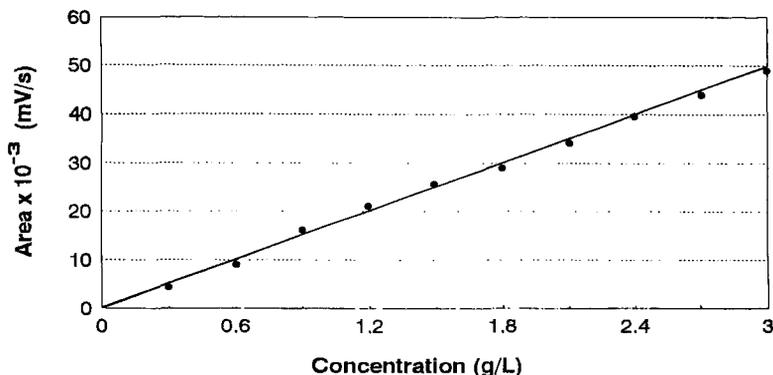
FIGURE 5.
Calibration graph for the determination of tartaric acid
eluted according to the method described.

detector was linear in the tested range and linear
regression analysis yielded y = 16.33 x + 0.31 with a
correlation coefficient of 0.9989 (Fig. 5).

The detection limit for tartrate, defined as
signal-to-noise ratio of 3:1, was 12 µg/mL.

Recovery experiments were carried out by adding known
amounts of tartaric acid to aliquots of two different wines.
Tartaric acid was spiked at six different concentrations for
Clos Reginu and Poggese wines. Typical recoveries,
determined by comparing the found and calculated amounts of
tartrate after spiking, are reported in Table 1. Recoveries
ranging from 100 to 103% were obtained for Poggese wine.
Tartaric acid concentrations (± S.D.; nine independent
measurements) in Clos Reginu and Poggese wines were 1.19 (±
0.13) g/L and 2.71 (± 0.07) g/L, respectively.

Table 1. Recovery of tartrate from wine.

Amount in wine (g/L)	Amount added (g/L)	Amount calculated (g/L)	Amount found (g/L)	Recovery ± SD (mean) (%)
1.191	0.2	1.391	1.323	95.1 ± 4.6
1.191	0.6	1.791	1.682	93.9 ± 4.4
1.191	1.2	2.391	2.543	106.4 ± 5.5
2.709	0.9	3.609	3.732	103.4 ± 5.3
2.709	1.8	4.509	4.578	101.5 ± 5.7
2.709	2.7	5.409	5.412	100.1 ± 5.1

Recovery = (Amount found / amount calculated) x 100.

Of the common acidic components in wine, none have retention characteristics such that they could interfere with the tartrate peak. However, sometimes a peak of unknown identity occurs, which is only partially resolved from the tartrate peak (Fig. 3). In order to evaluate the extent of background interference, it is advisable to use a diode-array detector. Comparison of spectra, obtained at the leading edge, apex and trailing edge of the tartrate peak, can afford a purity check.

To evaluate the precision of the method, within-run and between-run coefficients of variation were calculated at four different concentrations of tartaric acid. The within-run precision, performed by repeated injections (n = 9) of aliquots of the same wine sample with 1.50 g/L of

Table 2. Precision of the assay for tartrate in wine.

Nominal concentration (g/L)	Concentration found (mean ± SD)	C.V. (%)
Within-run variation		
0.30	0.31 ± 0.02	6.45
0.60	0.62 ± 0.03	4.84
1.50	1.54 ± 0.07	4.55
3.00	3.04 ± 0.12	3.95
Between-run variation		
0.30	0.31 ± 0.01	3.23
0.60	0.61 ± 0.03	4.92
1.50	1.52 ± 0.06	3.95
3.00	3.03 ± 0.24	7.92

tartrate, was 4.55%. The between-run precision, obtained from analyses of the same sample repeated on five subsequent days, was 3.95%. The variations for spiked samples are presented in Table 2.

The concentrations of the major carboxylic acids determined by HPLC analysis of different European wines are reported in Table 3. Tartaric acid exhibits values ranging from 0.72 g/L (Liebfraumilch) to 2.88 g/L (Rossese). The wines which contain the highest amounts of tartaric acid (more than 2 g/L) are: Rossese di Finale Ligure, Poggese, Cinque Terre, Cortese del Piemonte, and Beaujolais.

TABLE 3

Determination of Major Carboxylic Acids in Wines by HPLC

Wine	Acid Concentration (g/L)				
	Tartaric	Malic	Lactic	Acetic	Tannic
Beaujolais (F)	2.21	1.74	1.19	-	0.42
Chablis (F)	1.35	0.24	2.61	-	0.01
Cinque Terre (I)	2.61	0.81	1.22	-	-
Clos Reginu (F)	1.19	0.88	2.85	-	0.31
Cortese (I)	2.56	0.56	1.86	-	0.02
Dolcetto (I)	1.86	-	-	0.86	0.32
Liebfraumilch (D)	0.72	1.91	2.84	-	0.01
Malaga (E)	0.97	1.27	0.91	-	0.06
Moscato (I)	0.89	2.41	-	0.73	-
Nebbiolo(I)	1.44	-	1.22	-	0.25
Pigato (I)	1.32	1.05	0.47	0.25	-
Poggese (I)	2.71	0.91	-	-	-
Rosé (GR)	1.78	0.55	3.12		0.14
Rossese (I)	2.88	-	1.34	-	0.37
Santorini (GR)	1.52	0.73	0.61	-	-
Sherry (E)	1.96	0.93	3.95		0.03
Vermentino (I)	1.91	-	2.66	0.21	-

DISCUSSION

Some single acids present in wines can be determined by standard methods (14,15). Tartaric acid concentration can be calculated by the Blouin-Rebelein method based on the reaction with ammonium metavanadate (15) or by gas-chromatographic methods (7,8). Higher values obtained by the colorimetric method with respect to the chromatographic method were assigned to a specific redox interaction of metavanadate ion with the vicinal diol moiety present in tartaric acid and in several other wine components (1).

A simple ion chromatographic method was proposed for the determination of tartrate concentration in urine (3). However, one disadvantage of the method was interference of sulfates, so that elimination of sulfates was a necessary pre-treatment step prior to the chromatographic separation. Under the operating conditions proposed in this paper no problem exists, because absorbance of sulfates at 210 nm is negligible.

Although the main aim of this study was the determination of the tartaric acid concentration in wines for the reasons of medical interest mentioned above, the HPLC analysis described permits determination of other carboxylic acids important to study the organoleptic properties of a wine. In fact, as the choice of a wine is affected also by the organoleptic properties, these can be related to the relative amount of malic, lactic and tannic acids. The best characteristics for a wine are given by a lower quantity of malic acid together with a higher concentration of lactic acid; tannic acid must be present in a moderate amount (16). With regard to this, the most appreciated wines result those presenting a good ratio tartaric acid / malic acid. Since personal choices based on aspect, bouquet and taste, have to be taken into account, it is interesting to note that high amounts of tartaric acid seem to predominate in white rather than red wines. The amount of tannic acid confirms that this acid is present in good quantity in red wines.

The results of HPLC analyses are useful, when the composition of the diet has to be assessed, for choosing a wine with a high amount of tartaric acid, in order to prevent the formation of calcium stones.

The adaptation of reversed-phase HPLC to the determination of tartrate in biological fluids should be evaluated to investigate tartrate metabolism, e.g., the potential role of tartrate as an inhibitor of crystallization in calcium nephrolithiasis.

ACKNOWLEDGEMENTS

REFERENCES

1. E. Mentasti, M.C. Gennaro, C. Sarzanini, C. Baiocchi, M. Savigliano, J. Chromatogr., 322: 177-189 (1985).

2. V.S. Chadwick, A. Vince, M. Killingley, O.M. Wrong, Clin. Sci. Mol. Med., 54: 273-281 (1978).

3. M. Petrarulo, M. Marangella, O. Bianco, F. Linari, Clin. Chem., 37: 90-93 (1991).

4. P.C. Hallson, G.A. Rose, "Urolithiasis and related clinical research", P.O. Schwille, L.H. Smith, W.G. Robertson, W. Vahlensieck, eds., Plenum Press, New York, 1985, pp. 847-850.

5. B.K. Sur, H.N. Pandey, S. Deshpande, R. Pahwa, R.K. Singh, Tarachandra, "Urolithiasis: clinical and basic research", L.H. Smith, W.G. Robertson, B. Finlayson, eds., Plenum Press, New York, 1981, pp. 333-336.

6. F.P. Underhill, F.I. Peterman, A.G. Krause, J. Pharmacol. Exp. Ther., 43: 351-358 (1931).

7. P. Dietel, C. Holzel, K. Schneider, G. Spitteler, J. Chromatogr., 422: 1-12 (1987).

8. G. Hoffmann, S. Aramaki, E. Blumm-Hoffmann, W.L. Nyhan, L. Sweetman, Clin. Chem., 35: 587-595 (1989).

9. R. Schwarzenbach, J. Chromatogr., 251: 339-358 (1982).

10. G.O. Guerrant, M.A. Lambert, C.W. Moss, J. Clin. Microbiol., 16: 355-360 (1982).

11. C. Baiocchi, E. Campi, M.C. Gennaro, E. Mentasti, C. Sarzanini, Ann. Chim. (Rome), 73: 659-673 (1983).

12. M.J. Cooper, M.W. Anders, Anal. Chem., 46: 1849-1854 (1974).

13. E. Grushka, H.D. Durst, E.J. Kikta, Jr., J. Chromatogr., 112: 673-678 (1975).

14. L.U. Tomasset, Chimica Enologica, AEB, Brescia, 1978.

15. EEC Regulations, No. 2984/78, Brussels, 1978; Gazzetta Ufficiale della Comunita' Europea, Ed. Italiana, No. L 360, EEC Official Publications Department, Luxemburg, 1978.

16. E. Peynaud, "Enologia e tecnica del vino", Ed. AEB, Brescia, Italy, 1976, pp. 59-64.

COMPARISON OF METHODS EVALUATING LIPOPEROXIDATION IN PLASMA OF MALARIA PATIENTS

M. A. CARBONNEAU[1], D. SESS[1], E. PEUCHANT[1],
M. C. RECEVEUR[2], L. DUBOURG[1], M. J. THOMAS[1],
A. PERROMAT[1], AND M. CLERC[1]

[1]Laboratoire de Biochimie Médicale A. Université de Bordeaux II
146, rue Léo Saignat
33076 Bordeaux Cedex, France
[2]Service des Maladies Tropicales
Hôpital Saint-André
1, rue J. Burguet
33075 Bordeaux Cedex, France

ABSTRACT

Lipid peroxidation is believed to be involved in malaria and plasma of malaria patients may be damaged by reactive oxygen species This study compares determinations of malondialdehyde (MDA), vitamins A and E by high performance liquid chromatography, thiobarbituric acid reactive substances (TBA-RS) by fluorometric analysis and fatty acids by gas chromatography. During malarial infection, polyunsaturated fatty acids (PUFA), vitamins A and E decreased significantly when TBA-RS increased significantly. However, there was no significant change in total or MDA bound to amino-groups. On the other hand, there was no correlation between

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TBA-RS production and PUFA decrease or between TBA-RS production and vitamin A or E consumption. These results support the concept that other compounds such as proteins and cholesterol may be involved in the formation of these TBA-RS.

INTRODUCTION

Lipid peroxidation (LPO) is believed to be intimately involved in the aetiology of a wide range of diseases including malaria (1). Highly reactive oxygen species (ROS) produced by host mononuclear phagocytes activated during the natural course of malaria can be released extracellularly and have been shown to kill intra-erythrocytic parasites (2-4) by imposing oxidant stress (5,6). It has been suggested that some aspects of severe malaria may result from exaggerated ROS effects on host tissues (3). So plasma in close contact with the host-parasite interaction might be susceptible to ROS damage and LPO.

It is widely assumed that LPO is triggered whenever conditions of increased oxidative stress and/or decreased anti-oxidant defenses occur in plasma or tissues. Therefore, the aim of the present work was to investigate techniques capable of analysing plasma LPO, including high performance liquid chroma-tography (HPLC) determination of malondialdehyde (MDA : LPO end product), of vitamins A and E (lipophilic antioxidants) and fluoro-

metric determination of thiobarbituric acid reactive substances (TBA-RS : LPO breakdown products), and finally gas chromato-graphic (GC) determination of fatty acids (FA : LPO substrates). Results suggest that adequate assessment of LPO cannot be achieved by a single measurement of MDA or TBA-RS, and that knowledge of FA composition is required owing to the indirect and complex relationship between LPO and the TBA test.

MATERIALS AND METHODS

Blood samples

Blood samples were obtained from 28 adult malaria patients (men and women, age range 19-59 years) infected with *Plasmodium falciparum* (23 patients), *P. vivax* (2 patients), or *P. ovale* (3 patients). Malaria was diagnosed on the basis of clinical symptoms and a parasite-positive blood determination. All patients were in an acute phase and had not received any curative treatment. Malaria patients living in Europe were chosen to avoid including individuals carrying genetically-determined abnormalities (sickle cell diseases, thalassemia, glucose-6 phosphate dehydrogenase deficiency) that increase oxidant stress on the red cell and its contents (7).

Blood was collected by veinipuncture into vacutainer tubes (Becton Dickinson, Grenoble, France) with heparin after an

overnight fast. Parasites were counted in blood and expressed as percentage of infested red blood cells (RBC).

Blood was also taken from 30 adult control subjects (men and women, age range 25-60 years). None of the controls was a heavy smoker, had a history of recent acute disease, or was taking drugs.

HPLC analyses

Free malondialdehyde of plasma was measured as previously described (8) after protein precipitation by 7 % $HClO_4$ and reaction of the clear supernatant with thiobarbituric acid (TBA) at 100°C for 30 min.

For total MDA analysis, plasma was incubated with NaOH at pH13 and 60°C for 30 min. The hydrolysed sample was then acidified to pH < 1 with $HClO_4$. After centrifugation, the clear supernatant was treated with TBA as for free MDA determination.

The difference between total MDA and free MDA determinations was considered to be the bound MDA to amino groups of proteins or nucleic acids.

Under our conditions, the mean pH value after TBA reaction was 0.75 ± 0.07 (mean ± SD). We selected a pH < 1 to minimize TBA reaction with oxidized compounds other than MDA (9,10), and because the TBA reaction with actual MDA is not pH dependent (11).

HPLC separations were done on a C18 μ Bondapak 3.9x300 mm column (Waters) with a 60/40 (v/v) mixture of 10 mmol/l phosphate

buffer pH 5.8 and methanol as a mobile phase, with a flow rate of 1.5 ml/min. The fluorometric detector (Waters model 470) was set at excitation 515 nm and emission 553 nm with gain x 1000. 1,1,3,3 tetraethoxypropane (Aldrich-Chemie, Steinheim, Germany) was used as a standard and concentrations were calculated using a molar extinction coefficient of $1.57 \ 10^5 \ M^{-1}cm^{-1}$ as reported previously (8). A C18 μ Bondapak guard column was also used.

Vitamins A and E were measured as previously described (12). Briefly, proteins were precipitated with absolute ethanol containing 0.1 % ascorbic acid (wt/vol), and a mixture of retinol acetate and tocopherol acetate as an internal standard. The vitamins were subsequently extracted in hexane. This extract was concentrated and dissolved in methanol. HPLC separations were done on a C18 μ Bondapak $3.9 x 300$ mm column (Waters) with a 1.5 % deionized water/methanol mixture as a mobile phase. A spectrophotometric detector (Waters λ Max-481) was monitored at 290 nm. Vitamins were quantified using appropriate extinction coefficients with standards obtained from Sigma Chimie.

Fluorometric analysis

Thiobarbituric acid reactive substances (TBA-RS) which are a way of measuring lipid peroxidation, were estimated by the method of Yagi (13). Under these conditions, the mean pH value was 1.3 ± 0.5 (mean ± SD). Briefly, to 50 μl of plasma, 4.0 ml of 42 mmol/l

H_2SO_4 were added followed by 0.5 ml of 35 mmol/l phospho-tungstic acid (PTA). After centrifugation for 10 min at 1000 g the precipitate was resuspended in 2.0 ml of H_2SO_4 and 0.3 ml of PTA and centrifuged again. The resulting precipitate was suspended in 4.0 ml of distilled water and 1 ml of 1 % TBA/pure acetic acid (v/v) was added. The mixture was heated for 1 h at 100° C and after cooling, extracted, with 5 ml of n-butanol. Fluorescence (Jobin et Yvon JY3 spectrofluorometer) in the butanol layer was measured at 515 nm excitation and 553 nm emission (band width 10 nm). 1.1.3.3 tetraethoxypropane was used as a standard without preliminary acid hydrolysis (8,14) and plasma TBA-RS concentrations were calculated using a molar extinction coefficient of $1.510^5 M^{-1} cm^{-1}$ (8).

Aldehydic compounds (trans 2-hexenal, trans 2-octenal, trans 2,4-hexadienal, trans 2,4-nonadienal...) react with TBA giving yellow 450 nm-, orange 495 nm- and red 532 nm-absorbing chromophores. Moreover this TBA reaction is pH dependent. At pH <1 (HPLC method of MDA analysis), the yield of the red 532 nm-absorbing pigment produced by these aldehydes at 100°C for one hour was only 0.03 to 0.3% of that of actual MDA, while the yield was 0.4% at pH 1.3 (fluorometric method of TBA-RS analysis) and 5-10% at pH 3.5 (15).

Gas chromatography (GC) analyses

Fatty acid composition was determined from 1 ml of the lipid extract after transformation into isopropylic esters (16).

Fatty acid esters were separated in a Carlo-Erba 6000 chromatograph equipped with a 25 m capillary column (0.32 mm internal diameter Carbowax). Column conditions were 180°C for 5 min, then rising by 7.5°C/min to 220°C for 35 min. The injector was at 60°C and the flame ionization detector at 250°C. Helium was used as a carrier gas (flow rate 2 ml/min). Peak identifications were made by comparison with reference fatty acids (Sigma Chimie) and peak areas were measured with an automatic integrator DP 700 Carlo-Erba. Quantification of each fatty acid was expressed as a percentage of the total extract.

Quantitative analyses of the two major PUFA, linolenic (omega-6 C18 : 2) and arachidonic (omega-6 C20 : 4) acids were done using the external standardization method. Peak area ratios were used for calculation following the internal standard method with pure arachidic methyl ester (Sigma Chimie) as internal standard.

Peroxidation of fatty acid standards

For this experiment, a mixture of 2 µmol of C18 : 2 methyl ester and 0.4 µmol of C20 : 4 methyl ester was ultrasonicated in 25 ml of phosphate buffer pH 7.4 at 0°C under N_2 and for 10 min. Lipid peroxidation was induced either by 10 µM $CuSO_4$ or by 10 mM H_2O_2 and 200 µM $FeSO_4$ and was allowed to proceed for 15 h at 37°C. The amounts of MDA and TBA-RS produced were then analyzed as previously described and expressed as nmol/ml of incubation mixture.

Statistical analysis

Data are presented as means ± SD or S.E.M. (details below). For comparison between healthy controls and the malaria population, Student's t-test was chosen and P < 0.05 was considered significant. Linear regression analysis was assessed by Spearman rank correlation. These analyses were carried out on a computer using a statistical software package (Stat-View).

RESULTS

All malaria patients were in an acute phase and had not received any curative treatment. They were all slightly anemic as shown by a significant (P < 0.025) decrease in RBC (Table 1). The amount of total plasma proteins was not significantly different in the two groups. Triglycerides were significantly (P < 0.01) increased when total cholesterol was significantly (P < 0.001) decreased in malaria patients compared to controls (Table 1).

Fatty acid composition of plasma lipids

Fatty acid (FA) composition was analysed by GC of isopropylic esters and an unsaturation index (UI) was calculated as the ratio of the sum of polyunsaturated FA percentages to the sum of saturated and monounsaturated FA percentages. Results are shown in Table 1. The unsaturation index was significantly

TABLE 1

Biochemical data of blood in healthy subjects
and malaria patients

| | Erythrocytes | Plasma | | | |
	(10^6/mm^3 of blood)	Proteins (g/l)	Triglycerides (mmol/l)	T – chol (mmol/l)	UI (mmol/l)
Healthy subjects (n=16)	5.10 ± 0.17	64.6 ± 0.1	0.96 ± 0.09	5.31 ± 0.09	0.77 ± 0.02
Malaria patients (n=28)	4.36 ± 0.14 $P < 0.025$	62.0 ± 0.9 NS	2.24 ± 0.32 $P < 0.01$	3.46 ± 0.17 $P < 0.001$	0.39 ± 0.04 $P < 0.001$

Results are expressed as means ± S.E.M.

NS : difference statistically not significant

T – chol : total cholesterol

UI : unsaturation index

decreased (P < 0.001) in malaria patients whatever the level of parasitaemia. This indicated without doubt an oxidation of polyunsaturated FA. Among polyunsaturated FA, only the main omega-6 ones, linoleic (C18 : 2), arachidonic (C20 : 4) and eicosatrienoic (C20 : 3) acids were significantly decreased (P < 0.005, P < 0.001 and P < 0.01 respectively) as shown in figure 1. Omega-3 docosahexaenoic acid (C22 : 6) was slightly decreased but not significantly so (P > 0.1) in spite of its high oxidation susceptibility index.

The two major PUFA in plasma, omega-6 C18 : 2 and C20 : 4 acids, were submitted to quantitative analysis in both groups. Results are shown in Table 2. During malaria ,the C18 : 2 and C20 : 4 decreases were then estimated to be 800 nmol/ml and 160 nmol/ml, respectively.

Analytical features of LPO product determination

To measure LPO product formation, we compared two analytical methods. The LPO products were simultaneously estimated after TBA reaction by HPLC allowing separation of the MDA/TBA adduct from other TBA-reactive substances (TBA-RS), and by fluorometric measurement of whole LPO products as previously proposed by Yagi. The first method made it possible to evaluate successively free and total MDA ; the difference between total and free MDA was considered to be the bound MDA. In the second method, TBA-RS other than lipoperoxides were eliminated
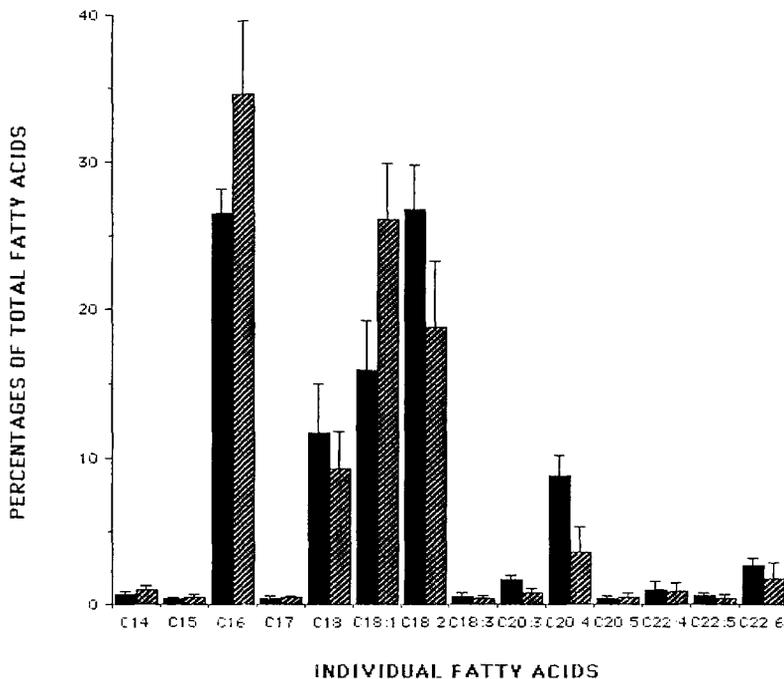
FIGURE 1 : Fatty acid profile in plasma of healthy subjects (■) and malaria patients (▨).
Loss in polyunsaturated fatty acids was statistically significant :
 ✳ P < 0.01; ✳✳ P < 0.005; ✳✳✳ P < 0.001.

by precipitating lipids and proteins with a mixture of phosphotungstic/sulfuric acid. The TBA reaction was then performed on the pellet. The level of LPO products can be expressed in terms of MDA and this value was considered to be the plasma TBA-RS. However, under these conditions, free MDA was not taken

TABLE 2

Decrease in plasma omega-6 C18 : 2 and omega-6 C20 : 4 acids
during malarial infection

	C18 : 2 (mmol/l)	C20 : 4 (mmol/l)
Healthy subjects	8.3 ± 1.9	0.44 ± 0.06
Malaria patients	7.5 ± 1.4	0.28 ± 0.03

Results are the means ± S.E.M. of 6 independent cases examined

into account. Therefore, we compared bound MDA measured by HPLC and TBA-RS value determined fluorometrically. The fluorescence detection used in both methods allowed more specific and sensitive detection. Results are shown in Table3. Bound MDA values were significantly lower (P < 0.001) than TBA-RS in both control subjects and malaria patients. Moreover, there was a significant (P < 0.05) positive correlation between bound MDA and TBA-RS value (r = 0.41; n = 24) in control subjects. On the other hand, the correlation was not significant (P > 0.1) in malaria patients (r=0.11; n = 26), as shown in figure 2.

Bound and total MDA values were not significantly different between the two groups whatever the level of parasitaemia. On the other hand, there was a significant increase (P < 0.001) in the TBA-RS of malaria patients compared to controls. However, there

TABLE 3

Peroxidation product and antioxidant concentrations

in plasma of healthy subjects and malaria patients

	Healthy subjects	Malaria patients		
		Total(a)	Infestation(b)	
			< 1 %	> 1 %
	(n=30)	(n=28)	(n=13)	(n=15)
TBA-RS (µmol/l)	1.89 ± 0.20	5.39 ± 0.34 P < 0.001	5.25 ± 0.41	5.53 ± 0.55
Bound MDA (µmol/l)	0.36 ± 0.006	0.28 ± 0.05 NS	0.30 ± 0.08	0.26 ± 0.07
Total MDA (µmol/l)	0.52 ± 0.05	0.39 ± 0.06 NS	0.42 ± 0.09	0.35 ± 0.08
Vitamin A (µmol/l)	1.99 ± 0.07	1.04 ± 0.16 P < 0.05	0.85 ± 0.16	1.18 ± 0.26
Vitamin E (µmol/l)	28.52 ± 1.87	16.69 ± 0.87 P < 0.05	16.37 ± 1.48	16.42 ± 1.10

Results are the means ± S.E.M.

(a) significance was calculated between healthy subjects and malaria patients

(b) difference between < 1 % – and > 1 % – infestation group means were not statistically significant
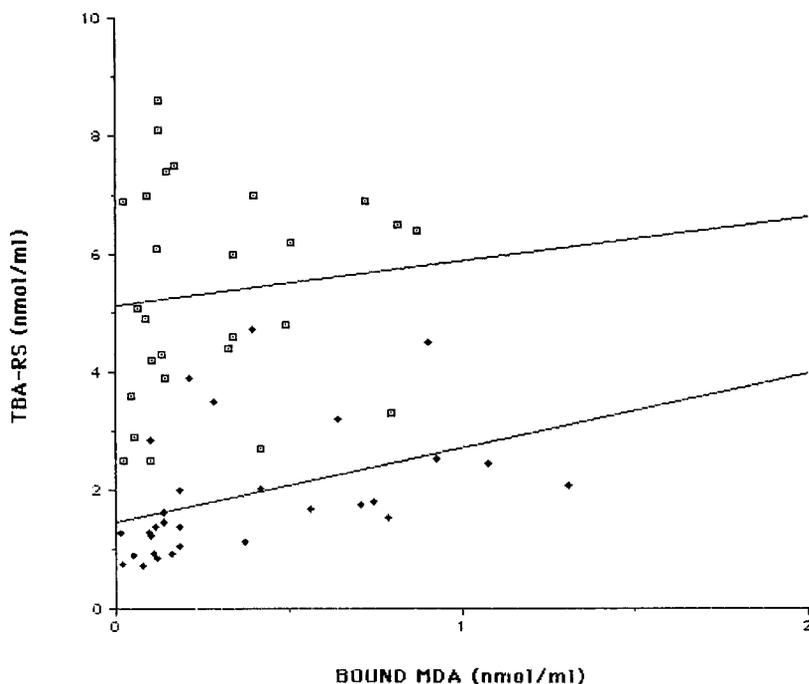
FIGURE 2 : Relationship between TBA-RS and bound MDA values
in (◆) healthy subjects (y=1.44 + 1.28 x ; r=0.41 ; P < 0.05) and
in (▫) malaria patients (y=5.13-0.75 x ; r=0.11 ; not significant).

was not a significant correlation between TBA-RS and the
percentage (z) of erythrocytes infested by *Plasmodium,* even after
Arc sin√z conversion in order to fit a gaussian distribution.

Figure 3 shows the correlations between plasma TBA-RS
values of healthy subjects or malaria patients and the fatty acid
unsaturation index (FAUI). Only the former showed a statistical
significance (r= -0.74 ; P < 0.01).
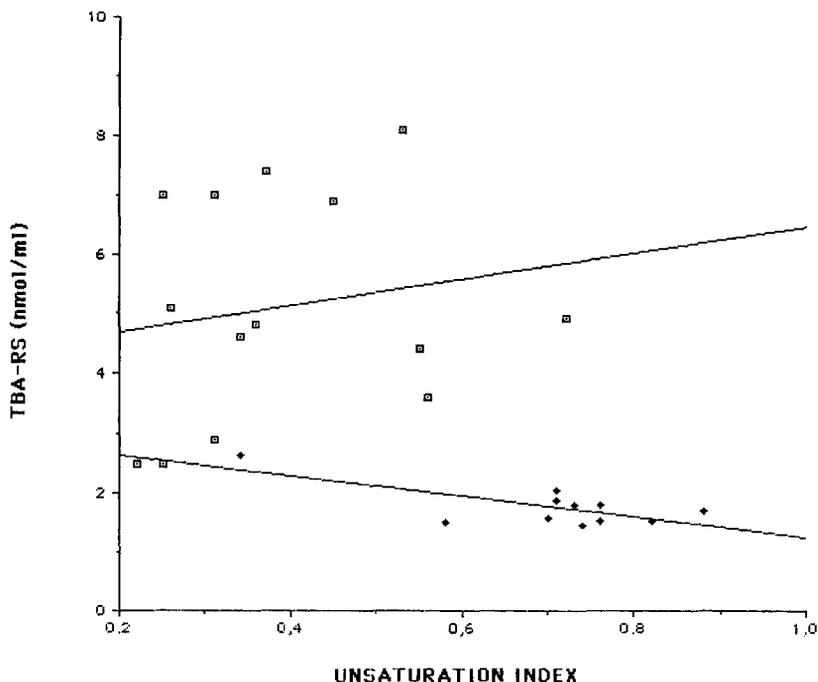
FIGURE 3 : Relationship between TBA-RS concentrations and unsaturation index
in ($\bullet$) healthy subjects (y=3.02-1.79 x ; r=-0.74 ; P < 0.01)
in ($\square$) malaria patients (y=4.24+2.24 x ; r=0.18 ; not significant).

Comparison of MDA and TBA-RS formation from plasma and pure fatty acids submitted to *in vitro* LPO

During the 15 h oxidation of a mixture containing 80 nmol/ml C18 : 2 and 16 nmol/ml C20 : 4 acids with either 10 μM Cu^{++}or 10 mM H_2O_2 and 200 μM Fe^{++} the pure fatty acids were totally disrupted (99.9-100 %) and produced the same TBA-RS level (3.52 ± 0.1 nmol/ml and 3.99 ± 0.07 nmol/ml respectively as shown

in Table 4. During Cu^{++} oxidation the MDA value was slightly lower (P > 0.1) than for TBA-RS. However, during H_2O_2/Fe^{++} oxidation, the former value was very significantly decreased (P < 0.001). Given the decrease in plasma PUFA during malaria infection and in *in vitro* peroxidation of the pure fatty acid reference, peroxidation products analyzed as MDA or TBA-RS could be compared. First, the decrease in PUFA during malaria infection did not increased the total MDA value (Table 3). Second, the TBA-RS production from *in vitro* LPO of the pure fatty acid preparation (Table 4) exceeded about 10-fold the increase in the TBA-RS value produced by malarial infection.

Plasma vitamin A and E status

There was a significant (P < 0.05) decrease in vitamin A and E content in malaria patients compared to controls. However, this decrease was not correlated with the percentage of infested erythrocytes, as shown in Table 3. Morever, the amount of vitamin A or E was not significantly correlated with the TBA-RS value, in either group.

DISCUSSION

It has been assumed that LPO is produced by mononuclear phagocyte activation during the natural course of malaria infection

TABLE 4

In vitro formation of MDA and TBA-RS during 15 h incubation in the presence of 10 µM Cu^{++} or 10 mM H_2O_2 and 200 µM Fe^{++} from a mixture of pure fatty acid methyl esters.

| | Cu^{++} oxidation | | H_2O_2/Fe^{++} oxidation | |
| | MDA | TBA-RS | MDA | TBA-RS |
	(nmol/ml)		(nmol/ml)	
Incubation mixture :	2.81 ± 0.23	3.52 ± 0.15	0.62 ± 0.04	3.99 ± 0.07
(80 nmol/ml C18: 2	NS		P < 0.001	
+ 16 nmol/ml C20:4)				

Results are the means ± S.E.M. of 5 independent experiments.

Differences were statistically significant between TBA-RS and MDA values in H_2O_2/Fe^{++} oxidation.

(1-6). This LPO was clearly proved by a significant decrease in omega-6 PUFA (C18 : 2, C 20 : 3, C20 : 4) in malaria patients as compared to controls. Omega-3 C22 : 6 acid sligity decreased but not significantly (figure 1). These results are in agreement with those of Bruna et al (17) who found that the *in vitro* oxidation rate of docosahexaenoic (C22 : 6) and eicosapentaenoic (C20 : 5) acids, two highly PUFA of the omega-3 series, were lower than the oxidation rate of linoleic (C18 : 2) and arachidonic (C20 : 4) acids, the PUFA of the omega-6 series.

On the other hand, total cholesterol significantly decreased ($P < 0.001$) whereas triglycerides increased ($P < 0.01$), indicating that cholesterol, like PUFA, is a basic lipid structure which could be susceptible to peroxidation (18). These results have been previously shown by Stocker et al (19) in plasma of mice infected by *Plasmodium vinckei*. The plasma triglyceride increase could be the result of increased levels of triacylglyceride-rich lipoproteins which are elevated in the plasma of animals infected with various strains of *Plasmodium* (20), thereby resulting in inhibition of lipoprotein lipase which suppresses triglyceride clearance (21). Stocker et al (19) have also shown a plasma cholesterol decrease paralleling that found in red blood cells, but of a smaller magnitude.

Additional evidence for the involvement of plasma LPO in malaria patients is the significant ($P < 0.05$) decrease in plasma vitamin A and E content as compared to control values (Table3),

indicating that this protective system was overwhelmed. However, this vitamin E decrease observed in plasma could be due to its transfer to membrane RBC noted by Stocker et al. (22) in response to an oxidative stress of extra- or intracellular origin during malarial infection, or could be partly due to its consumption as plasmatic antioxidant. A similar decrease in vitamin A was also observed by Thurnham et al (23) who associated this lower vitamin A value to the oxidative conditions present in plasma of malaria patients.

This study provides evidence for the occurence of LPO. However, total and protein-bound MDA concentrations determined by HPLC were not significantly different in the two groups. These findings suggest either that, at least, in plasma of malaria patients, LPO does not produce the end product that is MDA (24), or that MDA is rapidly oxidized and/or eliminated (25). In particular MDA is unstable in the presence of weak concentrations of hydrogen peroxide (15,26) which can be produced by the activation of macrophages by *P. falciparum* (27,28),

Our *in vitro* experiment of pure C18:2 and C20:4 acids submitted to LPO produced by H_2O_2/Fe^{++} revealed that a 10 mM concentration of H_2O_2 was sufficient to induce a significant decline (P < 0.001; Table 4) in the amount of MDA-TBA complexes formed as compared to the amount produced by 10 μM Cu^{++} oxidation. This decline in MDA formation may be due to its oxidation by H_2O_2. However, this oxidized MDA compound was able to

react with TBA, so the TBA-RS values were the same in both oxidation procedures.

We observed a highly significant increase ($P < 0.001$) in the amount of TBA-RS in malaria patients as compared to controls. However, the TBA-RS amount was not correlated with the percentage of infested erythrocytes, and the mean values at different parasitaemia levels were not altered significantly (Table 3). These results are in agreement with those of Mathews and Selvam (29) in *Plasmodium vivax* infected patients.

On the other hand, PUFA are the substrate required for MDA formation, and the amount of peroxidized lipids formed may be related both to the amount of substrate and to the level of peroxidation. So in healthy subjects who have a low level of peroxidation, there is a significant correlation between TBA-RS values and the FA unsaturation index (FAUI). So the greater the unsaturation index, the lesser the decrease in PUFA, and the lesser are the TBA-RS values (figure 2). Surprisingly, in malaria patients who have a high level of peroxidation, the correlation between TBA-RS values and the FAUI was not significant (figure 3).

In this TBA test, the red pigment may not reflect MDA alone but may reflect FA oxidized components including MDA, alk-2 enals, alka-2.4 dienals and hydroperoxide functions (30) or non-lipidic compounds such as glycoproteins and other molecules containing sugar components (24). Plasma precipitation with PTA-H_2SO_4 (13) considerably increased the TBA-RS amount obtained by this method

(31) with reference values in human plasma in the range of 3-4 mmol/l (32), compared to TBA-RS amounts using untreated plasma with values in the range of 0.4 to 0.9 mmol/l with (8,33,34) or without (35-37) HPLC separation. In this respect, Warso et al. (38,39) have demonstrated that 80 % of the TBA-RS in human plasma was found not to have originated from lipid hydroperoxide conversion to MDA, and so had no relationship with PUFA peroxidation. Hydroperoxide from linoleic acid peroxidation decomposes *in vitro* into various aldehydic products including MDA, but the yield of red pigment produced during the TBA reaction was only 0.4 % (40). Morever, at pH 1.3 used in Yagi's method of TBA-RS analysis, the yield of red pigment produced by aldehydes other than MDA was only 0.4 % of that of actual MDA (see Material and Methods), and could be considered as negligible (15). In malaria patients , the TBA-RS production was significantly ($P < 0.001$) increased but was about 10-fold lower than the *in vitro* production of pure FA preparation submitted to LPO.

Vitamin E appeared to be consumed (Table3) during malaria infection, and this consumption could be explained by the chain-breaking antioxidant effect of vitamin E which inhibits the chain reaction of lipid peroxidation between PUFA attack and oxidized compounds which are able to react with TBA. Morever, Flecha et al (41) have recently suggested that radical scavenging by tocopherols could be explained by an electron transfer between the phytyl side-chain and the chromanoxyl aromatic nucleus of

tocopherols through an electron transfer to the isoprenoid chain of carotenoids and ubiquinone. This hypothesis could explain the parallel vitamin A and E consumption observed in malaria (table 3). However, we are unable to show any significant correlation between vitamin A and E consumption and TBA-RS formation. This lack of correlation between vitamin A or E consumption and TBA-RS production may be due to protection by other antioxidants such as vitamin C (22,23) and enzymes such as glutathione peroxidase, superoxide dismutase and catalase (42).

This increased TBA-RS production which has no relationship with the loss in PUFA and the consumption in antioxidant vitamins supports the concept that other compounds such as proteins and cholesterol may contribute to the formation of these peroxidation products (43). In particular, total cholesterol was significantly lower in malaria patients. Cholesterol, like PUFA, could be susceptible to oxidation and these oxidized cholesterol compounds might act as a strong oxidant to emphasize TBA-RS formation. This hypothesis is at present under investigation in our laboratory.

Finally, the direct quantitative comparison between the loss in PUFA, the MDA amount specifically analyzed by HPLC, TBA-RS production and vitamin A and E consumption in a biological system such as human plasma takes into account the complex nature of the relationship between lipid peroxidation and the TBA test (24). As for the TBA-RS test in Yagi's method, it is better suited for the empirical indication of an oxidative injury.

REFERENCES

1 - I.A. CLARK, A.C. ALLISON, F.E. COX, Nature, 259 : 309-311 (1976).

2 - I.A. CLARK, N.H. HUNT, Infect. Immun., 39 : 1-6 (1983).

3 - I.A. CLARK, Parasitology Today, 3 : 300-305 (1987).

4 - R. STOCKER, N.H. HUNT, G.D. BUFFINTON, M.J. WEIDEMANN, P.H. LEWIS-HUGHES, I.A. CLARK, Proc. Natl. Acad. Sci. USA, 82 : 548-551 (1985).

5 - C.F. OCKENHOUSE, S. SCHULMAN, H.L. SHEAR, J. Immunol., 133 : 1601-1608 (1984).

6 - A.O. WOZENCRAFT, H.M. DOCKRELL, J. TAVERNE, G.A.T. TARGETT, J.H.L. PLAYFAIR, Infect. Immun., 43 : 664-669 (1984).

7 - I.A. CLARK, G. CHAUDHRI, W.B. COWDEN, Free Rad. Biol. Med., 6 : 315-321 (1989).

8 - M.A. CARBONNEAU, E. PEUCHANT, D. SESS, P. CANIONI, M. CLERC, Clin. Chem., 37 : 1423-1429 (1991).

9 - P.W. ALBRO, J.T. CORBETT, J.L. SCHROEDER, J. Biochem. Biophys. Met., 13 : 185-194 (1986).

10 - M.A. CARBONNEAU, A. PERROMAT, M. CLERC, Comptes-rendus du 8e Colloque International de Pont-à-Mousson, John Libbey Eurotext, London, Paris, 1993.

11 - M. MIHARA, M. OCHIYAMA K. FUKUZAWA, Biochem. Med., 23 : 302-311 (1980).

12 - A.M. MELIN, M.A. CARBONNEAU, M.J. THOMAS, M.J. MAVIEL, A. PERROMAT, M. CLERC, Food Additives Contaminants, 9 : 1-9 (1992).

13 - K. YAGI, Meth. Enzymol., 105 : 328-331 (1984).

14 – J.M.C. GUTTERIDGE, Anal. Biochem., 69 : 518-526 (1975).

15 – M.A. CARBONNEAU, M.C. DELMAS-BEAUVIEUX, J.L. GALLIS, E. PEUCHANT, A. PERROMAT, P. CANIONI, M. CLERC, J. Liq. Chromatogr., 16 : 1385-1417 (1993).

16 – E. PEUCHANT, R.G. WOLF, C. SALLES, R. JENSEN, Anal. Biochem., 181 : 341-349 (1989).

17 – E. BRUNA, E. PETIT, M. BELJEAN-LEYMARIE, S. HUYNH, A. NOUVELOT, Lipids, 24 : 970-975 (1989).

18 – G. BACHOWSKI, J.P. THOMAS, A.W. GIROTTI, Lipids, 23 : 580-586 (1988).

19 – R. STOCKER, W.B. COWDEN, R.L. TELLAN, M.J. WEIDEMANN, N.H. HUNT, Lipids, 22 : 51-57 (1987).

20 – P. MAUROIS, P. CHARET, A. NOUVELOT, J.C. FRUCHART, A. VERNES, J. BIGUET, Ann. Trop. Med. Parasitol., 74 : 17-28 (1980).

21 – P.J. HOTEZ, N. LE TRANG, A.H. FAIRLAMB, A. CERAMI, Parasit. Immun., 6 : 203-209 (1984).

22 – R. STOCKER, N.H. HUNT, M.J. WEIDEMANN., I.A. CLARK, Biochim. Biophys. Acta, 876 : 294-299 (1986).

23- THURNHAM, R. SINGKAMANI, R. KAEWICHIT, K.WONGWORAPAT, Br. J. Nutr., 64 : 257-271 (1990).

24 – D.R. JANERO, Free Rad. Biol. Med., 9 : 515-540 (1990).

25 – H.H. DRAPER, M. HADLEY, Xenobiotica, 20 : 901-907 (1990).

26 – P. KOSTKA, C.Y. KWAN, Lipids, 24 : 545-549 (1989).

27 – B.M. BABIOR, N. Engl. J. Med. 298 : 659-668 (1978).

28 – N. DE RUITER, H. MULIAWAN, H. KAPPUS, Toxicology, 17 : 265-268 (1980).

29 - J.T. MATHEWS, R. SELVAM, Biochem. Intern., 25 : 211-220 (1991).

30 - H. KOSUGI, K. KIKUGAWA, Free Rad. Biol. Med., 7 : 205-207 (1989).

31 - T. HENDRIKS, R. F.T.A. ASSMANN, Clin. Chim. Acta, 174 : 263-270 (1988).

32 - K. YAGI, Lipid Peroxides in Biology and Medecine Academic Press, New York, 1982.

33 - S.H.Y. WONG, J.A. KNIGHT, S.M. HOPFER, O. ZAHARIA, C.N. Jr LEACH, F.W. Jr SUNDERMAN, Clin. Chem., 33 : 214-220 (1987).

34 - I.S. YOUNG, G.R. TRIMBLE, Ann. Clin. Biochem., 28 : 504-508 (1991).

35 - H. KAPPUS, Oxidative Stress, H. Sies ed, Academic Press Inc. London, 1985.

36 - A. LEDWOZYN, J. MICHALAK, A. STEPIEN, A. KADZIOLRA, Clin. Chim. Acta, 155 : 275-284 (1986).

37 - C. JENY, J. GOY, J. FECHNER, J. LOEPER, J. EMERIT, La Presse Médicale, 15 : 1131-1133 (1986).

38 - M.A. WARSO, W.E.M. LANDS, Clin. Physiol. Biochem., 2 : 70-77 (1984).

39 - M.A. WARSO, W.E.M. LANDS, J. Clin. Invest., 75 : 667-671 (1985).

40 - H. KOSUGI, T. KOJIMA, K. KIKUGAWA, Lipids, 24 : 873-881 (1989).

41 - B.S.G. FLECHA, M. REPETTO, P. EVELSON, A. BOVERIS, Xenobiotica, 21 : 1013-1022 (1991).

42 – A.O. WOZENCRAFT, Parasitology, 92 : 559-567 (1986).

43 – O.M. PITKANEN, Scand. J. Clin. Lab. Invest., 52 : 379-385 (1992).

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC): AN IMPORTANT TECHNIQUE IN THE STUDIES OF LIGNOCAINE AND ITS METABOLISM

Y. CHEN AND JULIA M. POTTER

Department of Clinical Pharmacology
1st Floor Lions Building
Princess Alexandra Hospital
Woolloongabba, Qld 4102, Australia

ABSTRACT

The high performance liquid chromatography (HPLC) assays
which have been developed for the studies of lignocaine
over the past 15 years have been reviewed. Studies of
lignocaine and its metabolism have been benefited
greatly by utilizing this modern technique. The
sensitivity and process time of HPLC have been greatly
improved by modifications including the use of solid-
phase extraction for sample preparation. A further
modification in the assay of hydroxylated derivatives is
presented. In the study of lignocaine-monoethylglycine-
xylidide (MEGX) as a dynamic test of liver function,
HPLC assay possesses some advantages over fluorescence
polarisation immunoassay (FPIA).

INTRODUCTION

Lignocaine was synthesised by Löfgren in 1946 [1],

an important date in the development and field of local

anesthetics.   The chemical structure of lignocaine is an
aromatic group, 2,6-xylidine, coupled to diethylglycine
via an amide bond.   In man, lignocaine mainly undergoes
de-ethylation by the hepatic cytochrome P-450 IIIA4 to
form monoethylglycine-xylidide (MEGX) and
glycinexylidide (GX) [2].   However, in some animals like
the rat, the main metabolic pathway for lignocaine is
hydroxylation rather than de-ethylation, with 3- and 4-
hydroxy (3- and 4-OH) lignocaine and 3- and 4-OH-MEGX
being the main metabolites [3].

   Before the development of gas chromatography (GC),
colorimetric (methyl orange) [4] and enzyme assay [5-6]
were used for studies of lignocaine's metabolism.   In
the early 1960's, GC was established for the measurement
of lignocaine [7-9], and it played a very important role
in the initial studies of the metabolism of lignocaine
[10-16].

   High performance liquid chromatography (HPLC) was
developed in the 1960's [17].   However, its application
to lignocaine's metabolism and pharmacokinetics was
delayed to the end of 1970's, becoming feasible with the
development of so-called reversed-phase chromatography.
Generally speaking, compared with GC, HPLC possesses
many advantages in the measurement of drugs and their
metabolite(s) in biological samples [18].

This review is intended to (1) describe generally the application of HPLC to the study of lignocaine metabolism with the establishment of various HPLC assays and (2) particularly discuss the use of lignocaine and the measurement of its main metabolite, MEGX, as a dynamic test of liver function in liver transplantation. The test is based on the conversion of lignocaine to MEGX, the latter concentration being used as an index of liver function. This test necessitates the use of a highly sensitive MEGX assay. The advantages and disadvantages comparing HPLC and another popular assay, fluorescence polarisation immunoassay (FPIA), used in this clinical assay role will also be discussed.

THE DEVELOPMENT OF HPLC ASSAYS AND THEIR APPLICATION ON THE METABOLISM STUDIES OF LIGNOCAINE

De-ethylated Metabolites

The first published HPLC assay for the measurement of lignocaine was developed by Adams et al. in 1976 [19]. Two years later Narang et al. [20] established the first HPLC assay for the measurement of lignocaine and its two main metabolites, MEGX and GX. From that time HPLC gradually replaced GC and became the first choice for the simultaneous measurement of lignocaine and its metabolites in biological specimens [21-33].

Published HPLC assays for the determination of lignocaine and its metabolites utilize liquid-liquid or\and solid-phase extraction, ultraviolet (UV) or electrochemical detection, with bonded reversed-phase columns or a various bare (unbonded) silica gel column for separation and some chemicals or drugs have been nominated as the internal standard. Table 1. summarizes these major differences from the HPLC assays reported to the present time.

Hydroxylated Metabolites

The first HPLC assay for the measurement of 3-OH- and 4-OH- lignocaine and 3-OH-MEGX was developed in 1984 by Tam et al. [37]. With modification, it was capable of the simultaneous determination of most hydroxylated and de-ethylated metabolites of lignocaine in plasma and urine [38]. A similar assay was reported by a Japanese group [39]. During our studies of lignocaine metabolism in the pig (submitted for publication), the latter assay [39] was substantially modified to allow the measurement of 3-OH-lignocaine and 3-OH-MEGX. In the reported procedure [39], the internal standard, procaine, was added after the first step of organic solvent extraction prior to the acid back-extraction. The neutralized acid phase then was evaporated to dryness with the residue reconstituted with mobile phase. In our experience, the

TABLE 1

Summary of Reported HPLC Assays for Lignocaine and Its
Metabolites.

Item		Reference Number
Column	Reversed-phase	19-22,25,28-31, 32,34
	Silica gel	23,26
Detection	Ultraviolet (UV)	19-23, 25-35
	Electrochemical	24
Internal Standard	EMGX* Trimethoprim p-Chlorodispyromide Bupivacaine Procaine Tocainide	20-22, 25-27 34-35 29-30 24 19,28,31-32 33
Extraction	Liquid-liquid Dichloromethane Ethyl acetate Chloroform Hexane Isopropanol Tert-butyl methyl ether	19,22,26,30,34 21,25,28,31-32 20 20 20 29
	Solid-phase	[24,27,33,35]

*: EMGX= Ethylmethylglycinexylidide

evaporation procedure usually took more than five hours

at 40 °C, and in addition required a vacuum facility.

There were no validation data available in the reported

assay. We therefore modified this procedure in order to

improve assay precision and reduce the process time.

The assay now utilizes trimethoprim as the internal

standard which is added to the serum sample prior to the

organic solvent extraction. Following acid back
extraction, the neutralized aqueous phase is blown with
an air stream until the residues of organic solvent are
completely driven off; this usually takes only two
hours. The aqueous phase is then injected directly into
the column. The standard curves of 3-OH-lignocaine
between concentrations of 10 to 2000 μg/L and 3-OH-MEGX,
20 to 350 μg/L, were linear with correlation
coefficients all more than 0.999. The coefficients of
variation of 3-OH-lignocaine at concentrations of 15,
100, 1600 μg/L and 3-MEGX at 25 100, 300 μg/L were all
less than 10%. Therefore, in our modified assay for the
measurement of 3-OH-lignocaine and 3-OH-MEGX, the
precision is ensured by adding internal standard before
the extractions and the requirement of both vacuum
evaporation and reconstitution become unnecessary.

    Compared with alternative assays available for the
de-ethylated metabolites of lignocaine (such as FPIA),
HPLC assay has an obvious advantage for having the
capacity to be modified as described for measurement of
its hydroxylated metabolites. This unique suitability
of the HPLC technique to be used for simultaneous assay
of multiple metabolites will ensure it an important role
in comprehensive studies on future lignocaine
metabolism.

HPLC ASSAY IN THE LIGNOCAINE-MEGX DYNAMIC LIVER FUNCTION TEST

Liver Function Test in Liver Transplantation

The rapid progress made in clinical liver transplantation has produced a climate conducive to the development of new and alternative tests of organ function other than those traditionally used. The predominant demands are that it be a sensitive and reproducible assay and that clinical relevance be demonstrated. Lignocaine and MEGX is being proposed in this major role. The first requirement of the MEGX assay, i.e the assay must be sensitive and reproducible, is established; the second, its clinical role, is currently under investigation.

In the area of liver transplantation, three different clinical facets are being considered. First, there is assessment of potential liver donors [40-44]; in this case the test should be readily available at any hospital with a potential donor; turnaround time should be rapid (e.g. within one hour) and there should be good sensitivity as an indicator of current liver function. Secondly, this test may be used to assess the severity of chronic liver disease and may in the future, help to determine the appropriate time for transplantation [45].

Thirdly, once the liver has been transplanted, this test
can be used to monitor the recipient with a good
specificity and sensitivity. Clinical events which have
been documented include primary non function [46],
hepatic artery thrombosis [47], ischemia [47], rejection
[42] and the general clinical course [48].

The ability of the liver to detoxify xenobiotics
has been promoted previously as the basis of tests used
to monitor liver function. Administration of particular
drugs/compounds with subsequent monitoring of their
elimination from serum has been studied for many years.
Generally speaking, these tests have one or more
deficiencies, notably a slow turnaround time, technical
complexity, and poor specificity and sensitivity [36,
49].

In 1987, Oellerich and colleagues noticed that the
rate of MEGX formation correlates with the rate of
Lignocaine clearance [40] and for the first time, they
suggested that the measurement of lignocaine metabolism
can be used to assess liver function.

Measurement of MEGX Concentration
Sensitivity is essential for any assay in the
measurement of MEGX concentration as an index of liver
function in liver transplant recipients and end-stage

liver disease patients awaiting for transplantation. In
these patients, liver function is expected to be very
poor and the production of MEGX can be anticipated to be
very low. The detection limits of most published HPLC
assays [19-23, 25-33] are more than 20 μg/L, which is
obviously not sensitive enough [40-42, 45, 47].
Recently, we developed two HPLC assays [34-35] in which
the sensitivity was increased to 10 μg/L. They have
proved very useful for the measurement of MEGX
concentration in dynamic testing in liver
transplantation. The more recent method [35] utilized
solid-phase extraction for sample preparation. Various
packings were tested {C_{18}, C_8 and CN (1cc and 3cc) from
Waters (Millipore, Bedford, MA, USA) and Analytichem
Bond-Elut of C_8 (3cc), CN and phenyl (1cc) from Varian
(Harbor City, CA, USA)}. The 1cc phenyl Bond-Elut was
most appropriate for our needs. For optimum separation,
it is important to select suitable packing, but at the
same time attention must be paid to the volume of solid
phase, for this is critical for the sensitivity of the
assay, as it will determine the volume of final elution
solution.

Comparison of Fluorescence Polarisation Immunoassay
(FPIA) and HPLC

     FPIA has been used for the measurement of
lignocaine in serum samples for many years. However it

was not until recently that a FPIA assay for the
measurement of MEGX in serum specimens was developed
(Abbott Tdx).   According to the Production Information
[50] this assay has many advantages such as good
sensitivity and short process time which can meet the
demand for a dynamic liver function test in liver
transplantation.

    However, FPIA has some obvious disadvantages. (1)
The cost of an individual assay is expensive. (2) The
claimed limits of detection in the assay is 10 ng/ml,
however, this has not been supported by company CV data
to show that if the measured concentration at this low
level is reliable. (3) This assay is for MEGX only. For
any metabolic and pharmacokinetic study, it is
preferable if the concentration of parent drug is
measured simultaneously. In the particular case of the
MEGX test, the concentration of lignocaine provides
useful information to check whether the required dose of
lignocaine has been given. (4) FPIA is not suitable for
those samples with high bilirubin levels. Raised
bilirubin concentrations are common in liver
transplantation recipients early after transplantation
and in those end-stage liver disease patients who are
awaiting transplantation. In the presence of raised
bilirubin sample dilution is frequently needed and

therefore the process time is prolonged and sensitivity
decreased.

    In the newly developed HPLC assays [34-35], MEGX
sensitivity has been improved, the detection limit being
10 μg/L with a coefficient of variance (CV) < 4%. This
is particularly useful for the measurement of low MEGX
concentrations from patients with poor liver function.
Additionally, trimethoprim was utilized as internal
standard for the first time in a lignocaine-MEGX HPLC
assay. Trimethoprim is more readily available than the
previously most commonly-used internal standard,
ethylmethylglycinexylidide (EMGX).

    The claim for rapid assay of MEGX by FPIA is based
on a total process time of approximately 40 minutes for
20 samples [50]. It was the only assay with which the
MEGX results could be obtained within one hour.   All
previously reported HPLC assay using liquid-liquid
extraction [19-22,25,28-32,34] have long process times
involving time-consuming extraction and evaporation
procedures and in a further three HPLC assays using
solid-phase extraction [24,26,33], an evaporation step
was still used. However, in our HPLC assay using solid-
phase extraction [35], the final eluate can be directly
injected onto the column. Total time for extraction of

five serum/plasma samples is approximately 10 minutes
and HPLC run time for individual sample is about 10
minutes. In practice, that means that in an assay-run
incorporating a single point calibrator and two control
samples, the results of MEGX and lignocaine
concentrations from predose and 15-min samples from one
liver transplantation patient could be obtained within
one hour. This assay time is now comparable with what
only FPIA offered previously. This HPLC assay offers
not only an important alternative to the FPIA method
with comparable process time but also better
sensitivity. The limit of detection is 8 ng/ml. The
CV's for MEGX concentrations between 10 and 200 μg/L are
<9.5%. At the same time the simultaneous measurement of
GX can be achieved through minor adjustment of mobile
phase composition and flow rate. The limit of detection
of GX is 10 μg/L in this assay, the lowest one reported
to date.

    In the initial study by Oellerich [40] , the
comparison between HPLC and FPIA in the determination of
MEGX concentrations in patient's samples was in good
agreement with each other. Recently, another study [51]
compared MEGX concentrations by HPLC and FPIA in 119
samples from 45 liver transplantation donors and
recipients. The correlation coefficient between the two

methods was r=0.89. By using the technique of Bland and
Altman [52], the bias was found 12 μg/L towards FPIA
over the MEGX concentration range 0-250 μg/L. The major
difference between the two methods occurred in samples
from four recipients and one donor.   Significant cross
reactivity was not found in FPIA between MEGX and
lignocaine and two of its metabolites (GX and 2,6-
xylidine). Elevated plasma cholesterol and triglyceride
concentrations resulted in only relatively small
increase in apparent MEGX concentrations and whilst
there was an increase in apparent MEGX concentration, in
some samples with raised bilirubin concentrations, the
relationship was not constant.

<u>CONCLUSION</u>

This review has clearly shown that HPLC has found broad
and extensive application in studies of lignocaine
metabolism over the past 15 years. Its further
development is extending its utilization into other
areas. In the particular practice of the dynamic
testing of liver function in the field of liver
transplantation, HPLC has demonstrated its unique
advantages over FPIA. Undoubtedly, this modern
technique will be further developed expanded in other
areas in the future.

REFERENCE

1. N. Löfgren, Arkiv. Kemi. Mineral. Geol., 22A: 1-30 (1946)

2. M. J. Bargetzi, T. Aoyama, F. J. Gonzalez, A. Meyer, Clin. Pharmacol. Ther., 46: 521-527 (1989)

3. J. B. Keenaghan, R. N. Boyes, J. Pharmacol. Exp. Ther., 180: 454-463 (1972)

4. C. Y. Sung, A. P. Truant, J. Pharmacol. Exp. Ther., 112: 432-443 (1954)

5. G. Hollunger, Acta Pharmacol. Toxicol., 17: 365-373 (1960)

6. G. Hollunger, Acta Pharmacol. Toxicol., 17: 356-364 (1960)

7. A. H. Beckett, R. N. Boyes, J. B. R. Parker, Anesthesia 20: 294-298 (1965)

8. L. Kazyak, E. C. Knoblock, Anal. Chem., 35: 1448-1452 (1963)

9. J. Vessman, Acta Pharm. Suecica, 1: 183-198 (1964)

10. J. M. Strong, D. E. Mayfield, A. J. Atkinson, B. C. Burris, F. Raymon, L. T. Webster, Clin. Pharmacol. Ther., 17: 184-194 (1975)

11. J. M. Strong, M. Parker, A. J. Atkinson, Clin Pharmacol Ther, 14: 67-72 (1973)

12. G. Nyberg, B. Karlen, I. Hedlund, R. Grundin, C. Bahr, Acta Pharmacol. Toxicol., 40: 337-346 (1977)

13. A. H. Beckett, R. N. Boyes, P. J. Appleton, J. Pharm. Pharmacol., 18 suppl.: 76s-81s (1966)

14. K. K. Adjepon-Yamoah, L. F. Prescott, Br. Pharmacol. Soc., 47: 672p-673p (1973)

15. J. Blumer, J. M. Strong, A. J. Atkinson, J. Pharmacol. Exp. Ther., 186: 31-36 (1973)

16. G. D. Breck, W. F. Trager, Science, 173: 544-546 (1971)

17. R. W. Yost, L. S. Ettre, R. D. Conlon, Practical Liquid Chromatography--An Introduction, Perkin-Elmer Corp., CT, USA, 1980.

18. J. Chamberlain, <u>Analysis of Drugs in Biological Fluids</u>, CRC Press Inc., Florida, USA, 1985.

19. R. F. Adams, F. L. Vandemark, G. Schmidt, Clin. Chim., <u>69</u>: 515-524 (1976)

20. P. K. Narang, W. G. Crouthamel, N. H. Carliner, M. L. Fisher, Clin. Pharmacol. Ther., <u>24</u>: 654-662 (1978)

21. R. L. Nation, G. W. Peng, W. L. Chiou, J. Chromatogr., <u>162</u>: 466-473 (1979)

22. J. Hill, A. Roussin, J. Lelorier, G. Caille, J. Pharm. Sci., <u>69</u>: 1341-1342 (1980)

23. B. A. Bidlingmeyer, J. K. Del Rios, J. Korpi, Anal. Chem., <u>54</u>: 442-447 (1982)

24. M. K. Halbert, R. P. Baldwin, J. Chromatogr., <u>306</u>: 269- 277 (1984)

25. F. A. Luzzi, T. L. Wenger, J. K. Klinger, A. Barchowsky, H. C. Strauss, J. Chromtogr., <u>311</u>: 291-299 (1984)

26. K. Kushida, K. Oka, T. Suganuma, T. Ishizaki, Clin. Chem., <u>30</u>: 637-640 (1984)

27. Y. K. Tam, M. L. Yau, H. A. Sim, S. Tawfik, Clin. Invest. Med., <u>7</u>: A7 (1984)

28. R. Kawai, S. Fujita, T. Suzuki, J. Pharm. Sci., <u>74</u>: 1219- 1224 (1985)

29. H. R. Angelo, J. Bonde, J. P. Kampmann, J. A. Kastrup, Scand. J. Clin. Lab. Invest., <u>46</u>: 623-627 (1986)

30. H. F. Proelss, T. B. Townsend, Clin. Chem., <u>32</u>: 1311-1317 (1986)

31. Y. K. Tam, S. R. Tawfik, R. T. Coutts, M. R. Gray, D. G. Wyse, J. Chromatogr., <u>423</u>: 199-206 (1987)

32. L. F. Prescott, K. K. Adjepon-Yamoah, R. G. Talbot, Br. Med. J., <u>1</u>: 939-941 (1976)

33. R. Verbesselt, T. B. Tjandramaga, P. J. D. Schepper, Ther. Drug Monit., <u>13</u>: 157-165 (1991)

34. Y. Chen, J. M. Potter, P. J. Ravenscroft, J. Chromatogr., <u>574</u>: 361-364 (1992)

35. Y. Chen, J. M. Potter, P. J. Ravenscroft, Ther. Drug Monit., 14: 317-321 (1992)

36. T. J. Schroeder, J. J. Tasset, A. J. Pesce, Am. Assoc. Clin. Chem. Inc., 12: 7-13 (1990)

37. Y. K. Tam, M. L. Yau, H. A. Sim, S. Tawfik, Clin. Invest. Med., 7: A7 (1984)

38. Y. K. Tam, S. R. Tawfik, R. T. Coutts, J. Chromatog., 423: 199-206 (1987)

39. R. Kawai, S. Fujita, T. Suzuki, J. Pharmaceut. Sci., 74: 1219-1224 (1985)

40. M. Oellerich, E. Raude, M. Burdelski, M. Schulz, F. W. Schmidt, B. Ringe, P. Lamesch, R. Pichlmayr, H. Raith, M. Scheruhn, M. Wrenger, Ch. Wittekind, B. Klawunn, A. Knoke, B. Mues, M. Farle, J. Clin. Chem. Clin. Biochem., 25: 845-853 (1987)

41. M. Burdelski, M. Oellerich, A. Bornscheuer, N. Luebbe, B. Ringe, P. Lamesch, E. Raude, H. Raith, M. Scheruhn, G. Gubernatis, R. Pichlmayr, Transplant. Proc., 21: 2392-2393 (1989)

42. T. J. Schroeder, D. A. Gremse, M. E. Mansour, A, W. Theuerling, M. E. Brunson, F. C. Ryckman, F. J. Suchy, I. Penn, J. M. Alexander, A. J. Pesce, M. R. First, W. F. Balistreri, Transplant. Proc., 21: 2299-2301 (1989)

43. J. M. Potter, P. E. Hickman, G. Balderson, S. V. Lynch, R. Strong, Transplant. Proc., 24: 198-199 (1992)

44. G. Balderson, J. M. Potter, P. E. Hickman, Y. Chen, S. V. Lynch, R. Strong, Transplant. Proc., 24: 1960-1961 (1992)

45. M. Oellerich, M. Burdelski, B. Ringe, P. Lamesch, G. Gubernatis, H. Bunzendahl, R. Pichlmayr, H. Herrmann, Lancet, 1: 640-642 (1989)

46. J. M. Potter, M. Wright, S. V. Lynch, R. Strong, A. Henderson, G. Balderson, N. I. Walker, P. E. Hickman, Med. J. Aust., 158: 125-126 (1993)

47. J. M. Potter, P. E. Hickman, Y. Chen, A. Henderson, M. Wright, S. V. Lynch, R. Strong, Ther. Drug Monit., 15: 145 (1993)

48. J. M. Potter, P. E. Hickman, Y. Chen, C. Scott, S. V. Lynch, R. Strong, Abstract accepted by 5th Asian-Pacific Congr. of Clin. Biochem., Kobe, Japan (1991)

49. W. F. Balistreri, K. D. R. Setchell, Front Gastrointest. Res., 16: 220-245 (1989)

50. MEGX product information. Abbott Laboratories, North Chicago, IL, USA.

51. Y. Chen, J. M. Potter, Clin. Chem., 38: 2426-2430 (1992)

52. J. M. Bland, D. G. Altman, Lancet, 1: 307-310 (1986)

LIQUID CHROMATOGRAPHY CALENDAR

1994

MAY 8 - 13: HPLC '94: Eighteenth International Symposium on High Performance Liquid Chromatography, Minneapolis Convention Center, Minneapolis, Minnesota. Contact: Mrs. Janet Cunningham, Barr Enterprises, P.O. Box 279, Walkersville, MD 21793, USA.

MAY 23 - 25: International Symposium on Polymer Analysis and Characterization (ISPAC-7), Les Diablerets, Switzerland. Contact: Howard G. Barth, ISPAC Chairman, DuPont Company, Central Research & Development, P. O. Box 80228, Wilmington, DE 19880-0228, USA or Dr. M. Rinaudo, CERMAV-CNRS, B. P. 53X, 38041 Grenoble Cedex France.

JUNE 1 - 3: Joint 26th Central Regional/27th Great Lakes Regional Meeting, ACS, Kalamazoo and Huron Valley Sections. Contact: H. Griffin, Univ of Michigan, Ann Arbor, MI 48109, USA.

JUNE 1 - 3: International Symposium on Hormone & Veterinary Drug Residue Analysis, Congress Centre Oud Sint-Jan, Brugge, Belgium. Contact: Prof. C. Van Peteghem, University of Ghent, Laboratory of Food Analysis, Harelbekestraat 72, B-9000 Ghent, Belgium.

JUNE 5 - 7: VIth International Symposium on Luminescence Spectrometry in Biomedical Analysis - Detection Techniques & Applications in Chromatography and Capillary Electrophoresis, Congress Centre Oud Sint-Jan, Brugge, Belgium. Contact: Prof. Dr. Willy R. G. Baeyens, University of Ghent, Pharmaceutical Institute, Dept. of Pharmaceutical Analysis, Harelbekestraat 72, B-9000 Ghent, Belgium.

JUNE 6 - 9: 8th International LIMS Conference, The Westin William Penn Hotel, Pittsburgh, PA. Contact: Richard R. Mahaffey, 8th Int'l LIMS Conference,

c/o Eastman Chemical Co., P. O. Box 1973/Bldg. 284, Kingsport, TN 37662, USA.

JUNE 12 - 15:   1994 Prep Symposium & Exhibit, sponsored by the Washington Chromatography Discussion Group, at the Georgetown University Conference Center by Marriott, Washington, DC.   Contact: Janet E. Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

JUNE 13 - 15: Fourth International Symposium on Field-Flow Fractionation (FFF'94), Lund, Sweden. Contact: Dr. Agneta Sjogren, The Swedish Chemical Society, Wallingatan 24, 2 tr, S-111 24 Stockholm, Sweden.

JUNE 17 - 19: 49th Northwest Regional ACS Meeting, Anchorage, Alaska. Contact: G. L. Trigiano, University of Alaska, 3890 University Lake Drive, Anchorage, AK 99508, USA.

JUNE 19 - 22:   68th Colloid & Surface Science Symposium, Stanford University, Stanford, California. Contact: Dept. of Chem. Engineering, Stanford, University, Stanford, CA 94305-5025, USA.

JUNE 19 - 22: 24th Northeast Regional ACS Meeting, Burlington, Vermont. Contact: W. R. Leenstra, Dept. of Chem., University of Vermont, Burlington, VT 05405, USA.

JUNE 19 - 23: 24th National Medicinal Chem. Symposium, Little America Hotel & Towers, Salt Lake City, Utah. Contact: M. A. Jensen & A. D. Broom, Dept. of Medicinal Chem, 308 Skaggs Hall, Univ of Utah, Salt Lake City, UT 84112, USA.

JUNE 19 - 24:   20th International Symposium on Chromatography, Bournemouth International Centre, Bournemouth, UK. Contact: Mrs. Jennifer A. Challis, The Chromatography Society, Suite 4, Clarendon Chambers, 32 Clarendon Street, Nottingham, NG1 5JD, UK.

JUNE 24 - 27: BOC Priestly Conference, Bucknell University, Lewisburg, PA, co-sponsored with the Royal Soc of Chem. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

JULY 31 - AUGUST 5: American Society of Pharmacognosy Annual Meeting, International Research Congress on Natural Products, joint meeting with the Assoc. Français pour l'Enseignement et la Recherche en Pharmacognosie, and the Gesellschaft fur Arzneipflanzenforschung, and the Phytochemical Society of Europe, Halifax, Nova Scotia, Canada. Contact: D. J. Slatkin, P. O. Box 13145, Pittsburgh, PA 15243, USA.

AUGUST 14 - 17: Summer National Meeting & Particle Technology Forum, AIChE, Denver, Colorado. Contact: AIChE Express Service Center, 345 East 47 Street, New York, NY 10017, USA.

AUGUST 21 - 23: Australasian Plastics & Rubber Inst. 7th Technology Convention, Melbourne, Australia. Contact: APRI, P. O. Box 241, Mont Albert 3127, Australia.

AUGUST 21 - 26: 208th ACS National Meeting, Washington, DC. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 29 - SEPTEMBER 2: Synthetic Membranes in Science & Industry, University of Tubingen, Germany. Contact: Dechema e.V., Exhibitions & Congresses, Theodor-Heuss-Allee 25, P. O. Box 150104, D-60486 Frankfurt am Main, Germany.

SEPTEMBER 4 - 9: 4th European Rheology Conference, Seville, Spain. Contact: C. Gallegos, Dept. de Ingenieria Quimica, Universidad de Seville, 41071 Seville, Spain.

SEPTEMBER 5 - 7: 25th International Symposium on Essential Oils, Grasse, France. Contact: Assoc. des Ingenieurs et Techniques de la Parfumerie (A.I.T.P.), 48 ave. Riou-Blanquet, 06130 Grasse, France.

SEPTEMBER 8 - 10: Surfaces in Biomaterials 1994, Scottsdale, Arizona. Contact: Surfaces in Biomaterials Foundation, c/o Ardel Management, P. O. Box 26111, Minneapolis, Minn, USA.

SEPTEMBER 12 - 15: 3rd International Conference on Separations in Biotechnology, University of Reading, U.K. Contact: SCI, Conference Office, 14/15 Belgrave Square, London SW1X 8PS, U.K.

SEPTEMBER 25 - 28: Future of Spectroscopy: From Astronomy to Biology, Ste.-Adele, Que., Canada. Contact: Doris Ruest, Conference Mgr., NRC Canada, Ottawa, Ont., Canada K1A 0R6.

SEPTEMBER 27 - 29: CCPC International Conference on Chemical Process Automation, Houston, Texas. Contact: AIChE Express Service Center, 345 East 47th Street, New York, NY 10017, USA.

OCTOBER 9 - 14: 186th Meeting of the Electrochemical Society. Contact: Electrochem. Soc., 10 S. Main St., Pennington, NJ 08534-2896, USA.

OCTOBER 16 - 19: 46th Southeastern Regional Meeting, ACS, Birmingham, Alabama. Contact: L. Kispert, Chem Dept, Univ of Alabama, Box 870336, Tuscaloosa, AL 35115, USA.

OCTOBER 17 - 19: 3rd Int'l Symposium on Supercritical Fluids, Strasbourg, France. Contact: Int'l Soc for the Advancement of Supercritical Fluids, 1 rue Grandville, B. P. 451, F-54001 Nancy Cedex France.

OCTOBER 18 - 22: 30th ACS Western Regional Meeting, Sacramento, California. Contact: S. Shoemaker, Calif. Inst of Food & Agricultural Res., Univ. of Calif-Davis, 258 Cruss Hall, Davis, CA 95616-8598, USA.

OCTOBER 23 - 27: Annual Meeting of the American Association of Cereal Chemists, Nashville, Tennessee. Contact: J. M. Schimml, AACC, 3340 Pilot Knob Road, St. Paul, MN 55121-2097, USA.

OCTOBER 24 - 26: 2nd Internat'l Conference on Chemistry in Industry, Bahrain, Saudi Arabia. Contact: SAICSC-ACS, P. O. Box 1723, Dhahrain 31311, saudi Arabia.

NOVEMBER 2 - 5: 29th Midwestern Regional Meeting, ACS, Kansas City, Kansas. Contact: M. Wickham-St. Germain, Midwest Res. Inst, 425 Volker Blvd, Kansas City, MO 64110, USA.

NOVEMBER 7 - 10: 13th Int'l Conference on the Application opf Accelerators in Research & Industry, Denton, Texas. Contact: J. L. Duggan, Univ. of N. Texas, Dept. of Physics, P. O. Box 5368, Denton, TX 76203, USA.

NOVEMBER 10 - 11: 17th Int'l Conference on Chemistry, Biosciences, Pharmacy & Environmental Pollution, New Delhi, India. Contact: V. M. Bhatnagar, Alena Chem of Canada, P. O. Box 1779, Cornwall, Ont., Canada K6H 5V7.

NOVEMBER 11 - 12: 3rd Conference on Current Trends in Computational Chemistry, Jackson, Mississippi. Contact: J. Leszczynski, Jackson State University, Dept. of Chem., 1400 J. R. Lynch St., Jackson, MS 39217, USA.

NOVEMBER 13 - 16: 50th Southwest Regional Meeting, ACS, Fort Worth, Texas. Contact: H. C. Kelly, Texas Christian Univ, Chem Dept, Ft. Worth, TX 76129, USA.

NOVEMBER 13 - 18: Annual Meeting of the A.I.Ch.E., San Francisco, California. Contact: AIChE Express Service Center, 345 East 47th St., New York, NY 10017, USA.

NOVEMBER 14 - 17: 33rd Eastern Analytical Symposium, Somerset, New Jersey. Contact: Sheri Gold, EAS, P. O. Box 633, Montchanin, DE 19710, USA.

NOVEMBER 16 - 18: Envirosoft'94, 5th Int'l Conference on Development and Application of Computer Techniques to Environmental Studies, San Francisco, California. Contact: Computational Mechanics, Inc., 25 Bridge St., Billerica, MA 01821, USA.

DECEMBER 4 - 6: IBEX'94, International Biotechnology Expo & Scientific Conference, Moscone Center, San Francisco, California. Contact: Cartlidge & Associates, Inc., 1070 Sixth Avenue, Suite 307, Belmont, CA 94002, USA.

1995

MARCH 6 - 10: PittCon'95: Pittsburgh Conference on Analytical Chemistry & Applied Spectroscopy, New Orleans, Louisiana. Contact: Pittsburgh Conference, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-9962, USA.

APRIL 2 - 7: 209th ACS National Meeting, Anaheim, Calif. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

APRIL 25 - 28: Biochemische Analytik '95, Leipzig. Contact: Prof. Dr. H. feldmann, Inst. fur Physiologische Chemie der Universitat, Goethestrasse 33, D-80336 Munchen, Germany.

MAY 31 - JUNE 2: 27th Central regional Meeting, ACS, Akron Section. Contact: J. Visintainer, Goodyear Research, D415A, 142 Goodyear Blvd, Akron, OH 44236, USA.

JUNE 6 - 8: 28th Great Lakes Regional ACS Meeting, LaCrosse-Winona Section. Contact: M. Collins, Chem. Dept., Viterbo College, La Crosse, WI 54601, USA.

JUNE 14 - 16: 50th Northwest/12th Rocky Mountain Regional Meeting, ACS, Park City, Utah. Contact: J. Boerio-Goates, Chem Dept, 139C-ESC, Brigham Young Univ, Provo, UT 84602, USA.

JULY 9 - 15: SAC'95, The University of Hull, UK, sponsored by the Analytical Division, The Royal Society of Chemistry. Contact: The Royal Society of Chemistry, Burlington House, Picadilly, London W1V 0BN, UK.

AUGUST 20 - 25: 210th ACS National Meeting, Chicago, Illinois. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 12 - 15: 5th International Symposium on Drug Analysis, Leuven, Belgium. Contact: Prof. J. Hoogmartens, Inst. of Pharmaceutical Sciences, Van Evenstraat 4, B-3000 Leuven, Belgium.

OCTOBER 18 - 21: 31st Western Regional Meeting, ACS, San Diego, Calif. Contact: S Blackburn, General Dynamics, P. O. Box 179094, San Diego, CA 92177-2094, USA.

OCTOBER 22 - 25: 25th Northeastern Regional Meeting, ACS, Rochester, New York. Contact: T. Smith, Xerox Corp, Webster Res Center, M/S 0128-28E, 800 Phillips Rd, Webster, NY 14580, USA.

NOVEMBER 1 - 3: 30th Midwestern Regional ACS Meeting, Joplin, Missouri. Contact: J. H. Adams, 1519 Washington Dr., Miami, OK 74354-3854, USA.

NOVEMBER 1 - 4: 31st Western Regional ACS Meeting, San Diego, California. Contact: T. Lobl, Tanabe Research Labs, 4450 Town Center Ct., San Diego, CA 92121, USA.

NOVEMBER 5 - 7: 30th Midwestern Regional Meeting, ACS, Joplin, Missouri. Contact: J. H. Adams, 1519 Washington Dr, Miami, OK 74354, USA.

NOVEMBER 29 - DECEMBER 1: Joint 51st Southwestern/47th Southeastern Regional Meeting, ACS, Peabody Hotel, Memphis, Tenn. Contact: P.K. Bridson, Chem Dept, Memphis State Univ, Memphis, TN 38152, USA.

DECEMBER 17 - 22: 1995 International Chemical Congress of Pacific Basin Societies, Honolulu, Hawaii. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

1996

FEBRUARY 26 - MARCH 1: PittCon'96: Pittsburgh Conference on Analytical Chemistry & Applied Spectroscopy, Chicago, Illinois. Contact: Pittsburgh Conference, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-9962, USA.

MARCH 24 - 29: 211th ACS National Meeting, New Orleans, LA. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

JUNE 16 - 21: "HPLC '96: Twentieth International Symposium on High Performance Liquid Chromatography," San Francisco Marriott Hotel, San

Francisco, California. Contact: Mrs. Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

AUGUST 18 - 23: 212th ACS National Meeting, Boston, Mass. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

OCTOBER 16 - 19: 52nd Southwest Regional ACS Meeting, Houston, Texas. Contact: J. W. Hightower, Dept. Chem. Eng., Rice University, Houston, TX 77251, USA.

OCTOBER 24 - 26: 52nd Southwestern Regional Meeting, ACS, Houston, Texas. Contact: J. W. Hightower, Chem Eng Dept, Rice Univ, Houston, TX 77251, USA.

NOVEMBER 6 - 8: 31st Midwestern Regional Meeting, ACS, Sioux Falls, South Dakota. Contact: J. Rice, Chem Dept, S. Dakota State Univ, Shepard Hall Box 2202, Brookings, SD 57007-2202, USA.

NOVEMBER 9 - 12: 48th Southeast Regional ACS Meeting, Greenville, South Carolina. Contact: H. C. Ramsey, BASF Corp., P. O. Drawer 3025, Anderson, SC 29624-3025, USA.

1997

APRIL 6 - 11: 213th ACS National Meeting, San Antonio, Texas. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 7 - 12: 214th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

1998

MARCH 29 - APRIL 3: 215th ACS National Meeting, St. Louis, Missouri. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 23 - 28: 216th ACS National Meeting, Orlando, Florida. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

1999

MARCH 21 - 26: 217th ACS National Meeting, Anaheim, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 22 - 27: 218th ACS National Meeting, New Orleans, Louisiana. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

2000

MARCH 26 - 31: 219th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 20 - 25: 220th ACS National Meeting, Washington, DC. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

2001

APRIL 1 - 6: 221st ACS National Meeting, San Francisco, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 19 - 24: 222nd ACS National Meeting, Chicago, Illinois. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

2002

APRIL 7 - 12: 223rd ACS National Meeting, Orlando, Florida. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 8 - 13: 224th ACS National Meeting, Boston, Mass. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

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University of North Carolina at Chapel Hill

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