Journal of Chromatography, 380 (1986) 109—116
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3144

AUTOMATIC PREPARATION OF HUMAN SERUM SAMPLES FOR ANALYSIS OF THE DRUG ENOXIMONE AND ITS SULPHOXIDE METABOLITE USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

J.D.H. COOPER and D.C. TURNELL*

Department of Biochemistry, Coventry and Warwickshire Hospital, Stoney Stanton Road, Coventry, CV1 4FH (U.K.)

(First received November 28th, 1985; revised manuscript received March 5th, 1986)

SUMMARY

A method for the estimation of the cardiotonic drug enoximone and its major sulphoxide metabolite, in serum, is described. The method uses a new technique for the preparation of biological material prior to the separation of analytes using high-performance liquid chromatography. This technique has been described as the automated sequential trace enrichment of dialysates (ASTED). The sample preparation process operates concurrently with the chromatographic separation. Aliquots of 500 μl of serum are required for the analysis of serum from patients who have received a single 75-mg dose of enoximone. WIthin- and between-run coefficients of variation were found to be 2.8 and 3.9% for enoximone and 2.5 and 3.7% for enoximone sulphoxide. These values were obtained from estimating serum supplemented with enoximone and its metabolite, at concentrations of 0.5 and 1.0 mg/l, respectively.

INTRODUCTION

The cardiogenic drug enoximone, 1,3-dihydro-4-methyl-5-[4-(methylthio)-benzoyl]-2*H*-imidazol-2-one, has been reported to have positive inotropic and vasodilatory activities [1], and its sulphur oxidation metabolite has also been shown to have similar pharmacological properties [2]. Previously described methods for the analysis of these two compounds in serum [2, 3] have involved a liquid—liquid extraction followed by high-performance liquid chromatography (HPLC) for the separation of enoximone (I) and its sulphoxide metabolite (II)*.

*Enoximone and enoximone sulphoxide were previously recognized as MDL 17 043 and MDL 19 438, respectively.

0378-4347/86/$03.50

In a previous communication [4] we described a concurrent process combining HPLC and sample preparation using the technique of automated sequential trace enrichment of dialysates (ASTED). This paper describes the application of this approach to the direct measurement of both compounds I and II in human serum. This method was developed primarily for pharmacokinetic studies of patients taking oral doses of the parent drug.

EXPERIMENTAL

High-performance liquid chromatographic unit

The gradient HPLC system used (Gilson International, Villiers-le-Bel, France) consisted of a Holochrome UV detector set at a wavelength of 335 nm and an absorbance scale setting of 0.05 a.u.f.s.; two 303/5SC HPLC pumps; an Apple IIe microcomputer using the Gilson 704 system manager (version 2.1) to control the gradient program; and a 620 Data Master for peak-area integration. The Apple IIe computer was linked with the sample preparation unit as described previously [4]. The column was 100 × 4.6 mm I.D. packed with 5-μm Spherisorb ODS II (Phase Separations, Queensferry, U.K.).

Sample preparation unit

The system configuration was identical to that described previously [4]. The main elements of the unit consisted of:

Dialyser block. Two 30-cm path length dialyser blocks fitted with Cuprophan C-type membranes (Technicon, Basingstoke, U.K.) were connected in series. The membrane has a molecular mass cut-off of 10 000 Dalton.

Trace enrichment cartridge. The design and dimensions of the trace enrichment cartridge (TEC) has been described previously [5]. For this application, the TEC was packed with 20 mg of 5-μm Hypersil ODS (Shandon Southern Products, Runcorn, U.K.).

Operation of the sample preparation unit.

Samples were aspirated via a pump into the donor side of the dialyser block. When the donor channel was completely filled with sample the pump was switched off and the sample held static. At this point, the recipient solvent is pumped continously through the dialyser block and into the TEC situated in place of the loop on the Rheodyne 7010 HPLC injection valve. At a predetermined time, the Rheodyne valve is actuated to the inject position and the enriched analytes eluted from the TEC by the HPLC solvent. With the Rheodyne valve still in the inject position, both donor and recipient channels of the dialyser were purged. The injection valve was then switched back to the load position and the TEC was re-equilibrated with the recipient phase passing through the dialyser. The next sample was then taken and the cycle repeated. In this manner, samples were prepared sequentially and a sample is prepared during chromatographic analysis of the previous specimen.

Reagents

Compounds I and II were obtained from Merrell Dow Pharmaceuticals (Cincinnati, OH, U.S.A.). Unless otherwise stated, all other chemicals were

analytical grade and obtained from BDH Chemicals (Poole, U.K.). Water purified through activated carbon and an ion-exchange resin (Spectrum C system, Elga, High Wycombe, U.K.) was used for all reagent preparations.

Stock standard. Compound I (25 mg) and compound II (50 mg) were dissolved in 250 ml of methanol.

Working standards. A working standard containing 1.0 mg/l I and 2.0 mg/l II was prepared by diluting 0.5 ml of the stock standard to 50 ml with pooled drug-free human serum. These were stored at −20° C and found to be stable for at least three months.

Ammonium acetate buffer (500 mM). Ammonium acetate (38.5 g) was dissolved in 800 ml of water and the pH adjusted to 5.0 with glacial acetic acid. This was then made up to 1 l with water.

Chromatographic conditions

All solvents were filtered through a 0.45-μm Nylon filter (Anachem, Luton, U.K.) and degassed with helium before use. Solvent A was stock ammonium acetate—water (4:96). Solvent B was stock ammonium acetate—acetonitrile (Far-UV HPLC grade, BDH Chemicals)—water (4:70:26). The gradient program used was as follows: at 0 min, 15% B; at 2.5 min, 60% B in 4.0 min; at 6.5 min, 15% B in 0.5 min; 4 min allowed for re-equilibration; solvent flow-rate was 2.0 ml/min.

Sample preparation

Approximately 600 μl of serum were loaded into plastic cups. The timing diagram for the sample preparation process is shown in Fig. 1. A 10 m*M* solution of ammonium phosphate buffer was used to flow through the

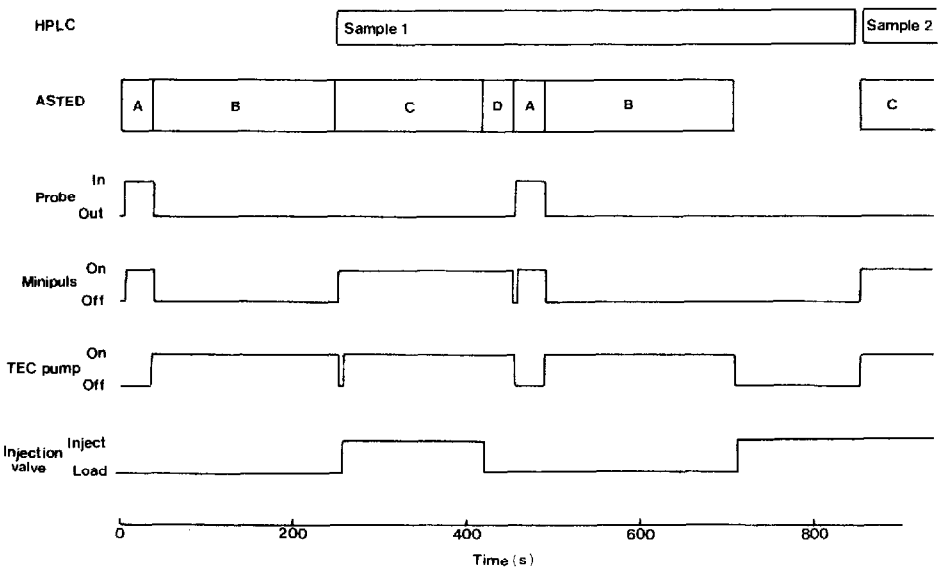

Fig. 1. Timing diagram showing the initial sample cycles of the operational sequence of events for the analysis of I and II in serum using the ASTED technique and HPLC in a concurrent mode. A = load sample; B = dialysis/trace enrichment; C = purge cycle; D = regeneration of TEC.

recipient side of the dialyser at a flow-rate of 2.5 ml/min. This flow-rate was maintained throughout the wash and sampling cycles.

Quantitation

Compounds I and II were identified in test sera by comparing their retention times to those obtained with a standard injection. Analyte concentrations in the test sera were calculated using the ratio of peak areas in the test chromatogram to the corresponding peak areas in the working standard chromatogram.

Quality control

Low and high controls were prepared by diluting 0.25 ml and 1.0 ml of the stock standard, respectively, to 50 ml with a drug-free human serum pool. This gave a low control with a value of 0.5 mg/l for I and 1.0 mg/l for II and a high control with a value of 2.0 mg/l for I and 4.0 mg/l for II. These two materials were analysed every ten samples to provide quality assessment data.

Single-dose studies of enoximone on hospitalized patients

This procedure was developed for the pharmacokinetic study of the cardiotonic properties of I. Part of the study consisted of an open trial during which patients suffering from congestive heart failure were given a single 75-mg dose of I. Blood was taken prior to the dose and at 1-h intervals for 6 h after the dose and assayed for both compounds I and II using the technique described. During this study, patients were maintained on their previous medication, which usually consisted of a combination of the diuretic frusemide, the antiarrhythmic drug amiodarone and digoxin.

RESULTS

Sample process time and chromatography

The trace enrichment times were chosen to obtain sufficient sensitivity for reliable estimation of the drugs. The injection valve was maintained in the inject position for 3 min to ensure complete removal of analytes from the TEC and eliminate carry-over.

Fig. 2 shows the chromatograms obtained using the procedure described. Thirty different sera were obtained at random, from patients not given this drug and processed through the system. No endogenous compounds were found to interfere with the assay. Also, the other drugs usually prescribed to our group of patients receiving enoximone did not produce any detectable peaks under the chromatographic conditions.

Precision, sensitivity, linearity and efficiency of method

Peak areas varied linearly with analyte concentration up to 3.0 mg/l for I and 6.0 mg/l for II. The sensitivity of the assay was established by analysing 30 drug-free sera after standardising the procedure with the calibration serum. The detection limit for each drug was calculated as the mean ± 2S.D. concentration of the zero standard peaks (n = 35). These were established for I as 0.010 mg/l and for II as 0.015 mg/l. The within-run coefficient of variation (C.V.) was estimated by assaying the low and high controls twenty times on the

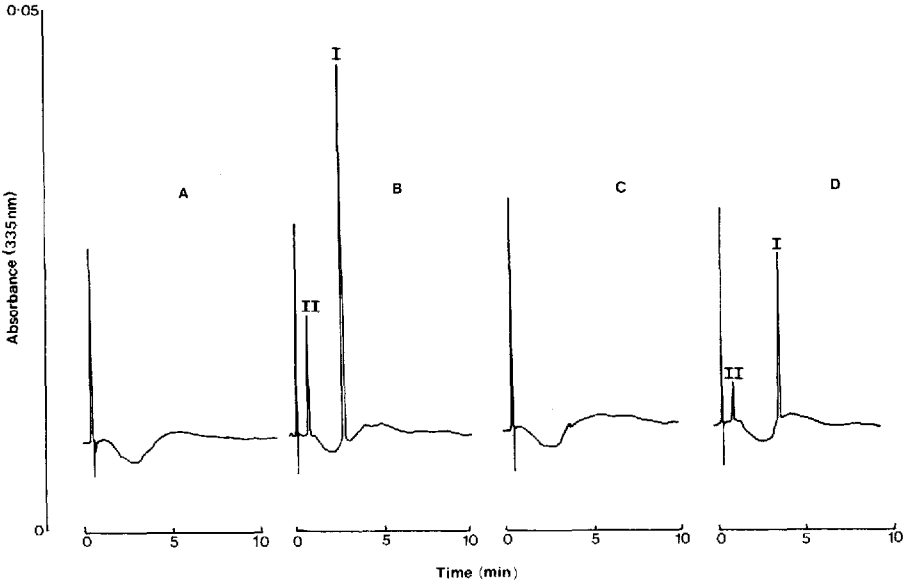

Fig. 2. Chromatograms of the following samples using the ASTED system described: (A) water sample; (B) pooled serum supplemented with 1.0 and 2.0 mg/l I and II, respectively; (C) same pooled serum before supplementing with drugs; (D) patients sera taken 2 h after a 75-mg dose of I; the concentrations of I and II were estimated to be 0.36 and 1.05 mg/l, respectively. I represents enoximone and II represents enoximone sulphoxide.

TABLE I

WITHIN- AND BETWEEN-RUN PRECISIONS OBTAINED BY ESTIMATING A POOLED SERUM SUPPLEMENTED WITH I AND II

The concentrations of analytes in high and low controls are stated in the text.

	Coefficient of variation (%)	
	Serum I	Serum II
Within-run		
Low control	2.8	3.5
High control	1.6	1.4
Between-run		
Low control	3.9	3.7
High control	2.5	2.0

same day. the between-run C.V. was obtained by estimating the same control samples twenty times on different days. The results are shown in Table I.

The efficiency of the system was determined by substituting the dialysis/TEC components with a standard 20-μl loop on the 7010 Rheodyne injection valve and injecting an aqueous working standard solution of I and II onto the HPLC column. The peak areas obtained were compared with those obtained after analysing the same working standard solution using the ASTED system. The recovery of analytes was calculated on the basis of the volume of

the donor side of the dialyser and found to be 54 and 50% for compounds I and II, respectively.

Matrix effect of serum samples on the estimation of enoximone and enoximone sulphoxide using the ASTED technique

A comparison was made of the peak areas obtained when sampling the calibration standard with those from an identical concentration of drugs diluted in 100 m*M* phosphate buffer (pH 7.4). The amounts of I and II determined in the serum calibration standard were 32 and 95% of the amounts of I and II found in the aqueous standard. To test for matrix differences between sera, samples were obtained from patients suffering from renal failure, nephrotic syndrome, hepatic and inflammatory disorders. Samples collected from healthy volunteers were also obtained. All the samples were spiked with stock standard to give a concentration of 1.0 and 2.0 mg/l for I and II, respectively. For the samples obtained from patients with pathological disorders, the mean concentration of I after assay was found to be 0.98 mg/l with a C.V. of 9.1% (n = 15), and for II the mean concentration was found to be 2.05 mg/l with a C.V. of 3.1% (n = 15). For samples obtained from healthy volunteers, the mean concentration of I after assay was found to be 0.97 mg/l with a C.V. of 3.8% (n = 15), and for II the mean concentration was 2.01 mg/l with a C.V. of 2.8% (n = 15).

Single-dose studies of enoximone on hospitalized patients

Fig. 3 shows the serum concentrations obtained for I and its metabolite at different times after one of the patients had orally ingested a single 75-mg tablet of I. Similar curves were observed for other patients taking this dose, but

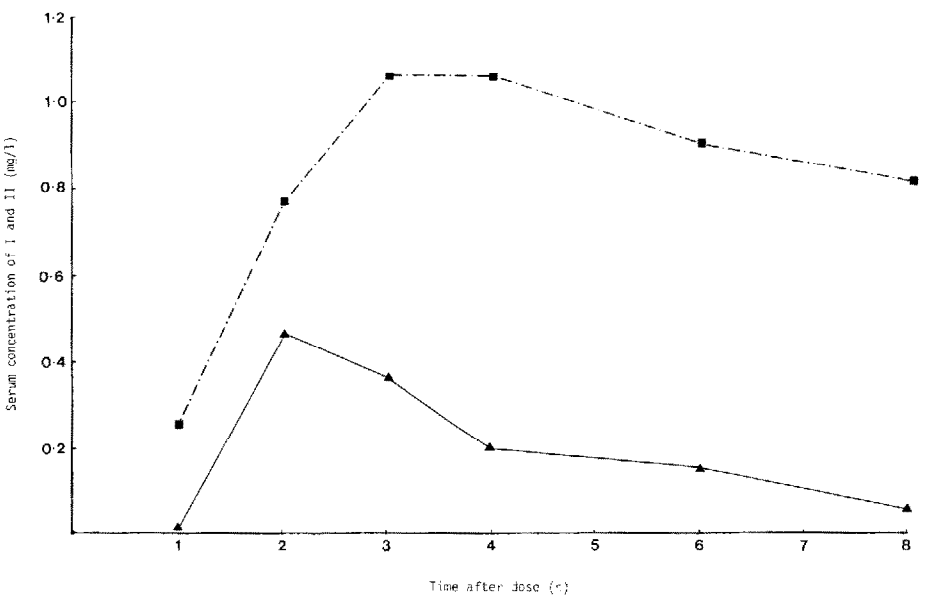

Fig. 3. Serum concentrations of I and II obtained after one of the patients had received a single 75-mg dose of I: ▲—▲ represents I; ■— · — · ■ represents II.

different peak concentrations in serum were found, ranging from 0.14 to 0.16 mg/l for I and from 0.71 to 1.31 mg/l for II.

DISCUSSION

The preparation of biological samples prior to HPLC analysis has always been a time-consuming process. The process described (ASTED) combined concurrently with HPLC has been shown to be a reliable and precise procedure for the estimation of both compounds I and II. Good precisions were obtained without the use of an internal standard (Table I). Because this type of sample preparation for biological fluids is extremely efficient and interference from fast-eluting compounds on the HPLC separation was negligible (Fig. 2), the time taken for the development of this method was extremely short.

Even though the movement of molecules across a semi-permeable membrane is not an efficient process in terms of rate of transfer, it has been shown previously [5] that good recoveries of analytes can be achieved with this exhaustive approach to dialysis. In the method described, high sensitivities were obtained, considering that concentrations of I were detected in patient sera after single doses of the drug. However, to achieve this sensitivity, two dialyser blocks were connected in series, which held approximately 500 μl of serum. Theoretically, changes in the dialyser design could reduce this volume considerably. Work is now in progress to effect this improvement.

The preparation of biological samples using a combination of dialysis and trace enrichment has previously been described using isocratic chromatography [5]. In this procedure, the high wavelength of 335 nm adopted for the detection of these drugs enabled gradient conditions to be employed and, although baseline shifts were evident (Fig. 2), these were not so severe as to affect the peak integration. This was supported by the good precision obtained for the assay. Gradient conditions were used in order to avoid the long chromatography times that would have occurred in an isocratic reversed-phase system due to the different polarities of I and II.

Equilibrium dialysis procedures have been used [6] to study protein binding and to determine free analyte concentrations. In view of this, it was possible that different sera would influence the recovery of the drugs using the method described. It is also well known that different disease states affect the degree of binding [7, 8]. To test this possible effect on this procedure, compounds I and II were added to sera taken from patients suffering from disorders that are recognized to alter protein binding. Although the acidic nature of I would suggest that any binding would occur with albumin, sera obtained from patients with inflammatory rheumatic states were also used. These sera had raised levels of acid glycoprotein, which has the property of binding basic drugs [8]. The imprecisions obtained for this group of samples were compared with imprecisions obtained from samples spiked with I and II obtained from healthy volunteers. The higher imprecisions for I (C.V. 9.1%) obtained from this analysis group compared with the imprecisions observed with the "normal" group (C.V. 3.8%) would tend to confirm that matrix effects do occur. Further evidence to support protein binding effects can be seen from the differences in recovery between I and the more polar sulphoxide metabolite. As may be ex-

pected, the polar metabolite is less protein-bound than the parent compound and, with the higher recoveries obtained, fewer differences occurred between samples and better precisions were obtained. It is possible that the inclusion of reagents to serum, which compete with the drugs for the protein binding sites, could improve the precisions obtained between samples by increasing the rate of release of the drugs from protein and thus minimizing effects between different sera. It is also possible that this automated procedure is capable of measuring free analyte concentrations. For the present, however, calibration of this assay with pooled human sera spiked with I and II is sufficient to give accurate and precise results.

The use of this technique has provided a rapid approach to the pharmacokinetic examination of I and its major active metabolite. Sufficient sensitivity was obtained for the study of the oral ingestion of single doses of the drug, enoximone. Using a trace enrichment time of 3 min, the detection limit for I was found to be 0.01 mg/l. Increasing the time will decrease the detection limit. The trace enrichment time does, however, depend upon the retention volume of the analytes for the trace enrichment material in the cartridge. The use of trace enrichment, prior to this technique, has been impeded when sampling biological material. However, the efficiency of dialysis as a step for removing interfering compounds overcomes this difficulty. Furthermore, in this procedure over 750 samples were treated before the TEC required repacking. This approach to the preparation of samples prior to HPLC permits rapid method development, low costs, direct sampling of biological samples, short sampling times and has been shown to be a precise technique for the assay of enoximone and enoximone sulphoxide in serum.

ACKNOWLEDGEMENTS

We wish to thank Gilson International (Villiers-Le-Bel. France) for the loan of the HPLC apparatus and Anachem Ltd. (Luton, U.K.) for its maintenance. We wish also to extend our gratitude to Mr. R.W. Richardson for his continued support, and Miss N. Shearsby, Mr. J. McIntosh, Mrs C. Fuller, Mr. M. Lewis and Miss Y. Parker for their excellent technical assistance. We thank Dr. D. Lockhart of Merrell Dow for supplying the enoximone and Dr. S. Choraria of the Cardiology Unit, Walsgrave Hospital, Coventry, for supplying the samples.

REFERENCES

1 R.C. Dage, L.E. Roebel, C.P. Hsieh, D.L. Weiner and J.K. Woodward, J. Cardiovasc. Pharmacol., 4 (1982) 500.
2 K.Y. Chan, D.F. Ohlweiler, J.F. Lang and R.A. Okerholm, J. Chromatogr., 306 (1984) 249.
3 K.Y. Chan, J.F. Lang and R.A. Okerholm, J. Chromatogr., 272 (1983) 396.
4 D.C. Turnell and J.D.H. Cooper, J. Automatic Chem., in press.
5 J.D.H. Cooper and D.C. Turnell, J. Automatic Chem., 7 (1985) 181.
6 H. Kurz, H. Trunk and B. Weitz, Drug Res., 7 (1977) 1373.
7 J.C. McElnay and P.F. D'Arcy, Drugs, 25 (1983) 495.
8 K.M. Piafsky, Clin. Pharmacokinet., 5 (1980) 246.