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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND AMPEROMETRIC DETECTION OF 3-O-METHYL ISOPRENALINE SULPHATE: APPLICATION TO STUDIES ON THE PRESYSTEMIC METABOLISM OF *d*-ISOPRENALINE IN MAN*

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SUMMARY

A selective method for the determination of 3-O-methyl isoprenaline sulphate in human urine and blood plasma has been developed using reversed-phase high-performance liquid chromatography with amperometric detection. The sulphoconjugate was subjected to acidic hydrolysis and the liberated 3-O-methyl isoprenaline was isolated by organic extraction and conventional cation exchange. An internal standard of 3-O-methyl isoetharine was synthesized from commercially avilable isoetharine and used to correct for recovery losses. The assay was shown to be linear over the range 5 ng/ml to 20 μg/ml with a limit of detection of 2 ng/ml. The reliability of the analytical method was examined together with its applicability to in-vivo studies in man.

INTRODUCTION

The conjugation of phenolic compounds with sulphate is an important mechanism for the detoxification of xenobiotics and natural compounds [1]. The process is intimately linked to the ingestion of food, which can contain the sulphate precursors cysteine and methionine (either free or in the form of protein) [2] and vitamin A, which is required for the activation of inorganic sulphate to 3-phosphoadenosine-5-phosphosulphate (PAPS) in a two-step reaction catalysed by ATP-sulphurylase (EC 2.7.7.5) and adenosine-5′-phosphosulphatephosphokinase (APS-phosphokinase) (EC 2.7.1.25) [3]. Phenolsulphotransferase (PST, EC 2.8.2.1) catalyses the transfer of sulphate from PAPS to

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an acceptor phenol substrate and has been identified in gut, liver, platelets, lung and kidney [4, 5].

Independent in-vitro evidence for the existence of two functionally distinct forms of human platelet PST has been presented [6, 7]. One form of the enzyme is relatively thermolabile (TL) [8] and catalyses the sulphate conjugation of monoamines (M) [6]. The other is thermostabile (TS) [8], catalyses the sulphoconjugation of micromolar concentrations of phenol and *p*-nitrophenol (P) and is selectively inhibited by 2,6-dichloronitrophenol [9].

It has been suggested that variations in platelet PST activity (measured in a blood sample) might reflect variations in PST activity in other organs such as the gut, which are more directly involved in sulphate conjugation and that such differences in activity might represent one factor contributing to individual variation in the sulphoconjugation of phenolic compounds [10—13].

As a result of in-vivo evidence for the existence of a sulphate conjugation defect [14—17] we selected *d*-isoprenaline (isoproterenol) as a model drug to investigate the possibility of an underlying polymorphism in gut sulphoconjugation [18]. Since isoprenaline is a PST (TL) or (M) specific substrate [19] and is conjugated exclusively with sulphate in man [20], it is likely to provide a clearer insight than that obtained with paracetamol (acetaminophen) [19—21], which is both a mixed TL—TS or M—P substrate and also a candidate for conjugation with glucuronic acid and sulphate in man[22, 23].

The variation observed in the sulphoconjugation of ingested *d*-isoprenaline [18], led us to speculate that low sulphate conjugation might be accompanied by increased O-methylation. In order to investigate this possibility, it was necessary to measure the 3-O-methyl metabolite of isoprenaline in plasma and urine. Previous analytical approaches have mostly used a cation-exchange clean-up step, followed by periodate oxidation and spectrophotometric measurement of the vanillin [24], ferricyanide oxidation and fluorimetric measurement of the trihydroxyindoles [25] or thin-layer chromatography (TLC) and scintillation spectrometry of tritium-labelled metabolites [26]. This paper describes the development and application of a reversed-phase high-performance liquid chromatographic (HPLC) assay with amperometric detection for the determination of 3-O-methyl isoprenaline sulphate in human plasma and urine.

MATERIALS AND METHODS

Apparatus

An Altex 100A HPLC pump provided a pulse-free flow of mobile phase to an Altex 210 injection valve fitted with a 100-μl loop and a 150 × 4.6 mm I.D. stainless-steel analytical column packed with 5-μm diameter Altex Ultrasphere octyl particles (Altex Scientific, Berkeley, CA, U.S.A.). The column was protected by a 50 × 2.1 mm I.D. stainless-steel pre-column hand packed with Whatman Co:Pell ODS (Whatman, Maidstone, U.K.). The electrochemical detection system comprised a Model LC-4 amperometric detector fitted with a TL-5 glassy carbon cell assembly (BioAnalytical Systems, West Lafayette, IN, U.S.A.). The column, detector and cell were enclosed in a Faraday cage of aluminium, itself earthed to the chart recorder, a Servoscribe RE 541.20 (Smiths Industries, London, U.K.).

Reagents

Organic solvents were all AnalaR grade, purchased from May and Baker (Dagenham, U.K.). S-Adenosyl-L-methionine (SAM) and glutathione (GSH) were obtained from Sigma (Poole, U.K.). Bio-Rex 70 cation-exchange resin was purchased from Bio-Rad Labs. (Watford, U.K.). Isoetharine hydrochloride 99% pure by TLC was a gift from Riker Labs. (Loughborough, U.K.). Catechol-O-methyltransferase (COMT, EC 2.1.1.6) was prepared from rat liver according to the method of Axelrod and Tomchick [27]. 3-O-Methyl isoprenaline 97% pure by TLC was supplied by Boehringer (Mannheim, F.R.G.). All other chemicals were of AnalaR grade and obtained from BDH (Chadwell Heath, U.K.).

Standards

A stock solution of 3-O-methyl isoprenaline (6 mg/ml) was prepared in 0.1 *M* hydrochloric acid and stored at 4°C. Subdilutions were made in 0.1 *M* hydrochloric acid and spiking achieved by adding 50 μl of appropriately diluted standard to 5 ml of drug-free urine or plasma.

Preparation of internal standard

No suitable internal standard was available commercially. From a consideration of the structure of 3-O-methyl isoprenaline, 3-O-methyl isoetharine was selected as a likely candidate internal standard (Fig. 1). It was synthesized from isoetharine using a ten-fold excess of SAM, in a reaction catalysed by partially purified COMT. The incubation mixture used was based on the recipe utilised in catecholamine radioenzymatic assays [28]. Initial time course experiments were performed as the yield of 3-O-methyl isoetharine at 1 h was very low. Conversion was found to be quantitative at 15 h incubating at 30°C and using 50 mg COMT preparation, 25 mg GSH, 6 ml Tris—magnesium chloride—EGTA buffer, pH 8.4, 1 ml of 0.5 mg/ml isoetharine and 1 ml of 5.0 mg/ml SAM. After incubation the pH of the solution was adjusted to below 1.0 with concentrated hydrochloric acid, the protein precipitate removed by centrifugation (2050 *g*, 4°C, 5 min) and the supernatant stored at 4°C.

Chromatographic separation

The mobile phase consisted of 0.1 *M* citrate—phosphate (McIlvaine) buffer

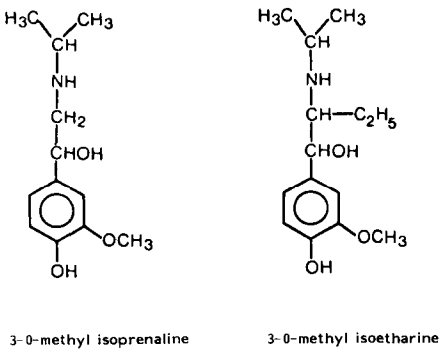

Fig. 1. Structural formulae of analytes.

of pH 3.2, containing 2.5 m*M* disodium EDTA and 11% methanol. This mixture was filtered through a 0.5-μm Gf/f glass microfibre filter (Whatman) and helium-degassed (BOC Special Gases, London, U.K.) prior to pumping at 1.6 ml/min through the HPLC system. Amperometric detection was achieved using a glassy carbon cell held at +0.80 V versus Ag/AgCl reference electrode. Typical sensitivities employed were 10 nA full scale for plasma and 250 nA full scale for urine.

Investigation protocol

Seven healthy volunteers (mean age 31.5 years) were asked to refrain from taking ascorbic acid and paracetamol for 12 h prior to dosing. On the day of the study no breakfast was taken but the subjects were allowed lunch 3 h after taking an oral dose of *d*-isoprenaline-*d*-bitartrate (2 mg/kg) in 100 ml of water. A baseline blood sample (10 ml) was taken into a chilled lithium heparin tube and further samples were taken at 30-min intervals up to 4 h, then hourly up to 8 h or in some cases 10 h. All blood samples were centrifuged at 2050 *g* for 15 min at 4° C and the separated plasma kept at −70° C until assayed. The protocol for this study was approved by the Research Ethics Committee of the Royal Postgraduate Medical School and Hammersmith Hospital and all subjects gave their informed consent. Two 24-h urines were collected.

Hydrolysis of sulphoconjugate

Acid-catalysed hydrolysis of 3-O-methyl isoprenaline sulphate was carried out as previously described [18]. Protein precipitation with perchloric acid was introduced prior to the plasma hydrolysis to avoid the possibility of gel formation on cooling. For plasma the procedure involved adding 250 μl of internal standard and 500 μl of concentrated perchloric acid to 3 ml of plasma in a polystyrene conical tube, standing for 5 min, mixing, centrifuging at 2050 *g* for 5 min at ambient temperature and then transferring the supernatant into heat resistant polyethylene tubes for a 30-min boiling period.

Extraction of 3-O-methyl isoprenaline

A 2-ml volume of toluene—isoamyl alcohol (3:2, v/v) was added to 2 ml of the acidic hydrolysate and the mixture shaken for 10 min (Denley Multivortex), followed by a brief centrifugation at 2050 *g*. The lower aqueous layer was frozen in a mixture of methylated spirit and solid carbon dioxide and the upper organic phase (containing organic acids) poured off. After thawing, the aqueous layer was tipped into a 20-ml plastic container (Sterilin) containing 10 ml of 4% boric acid and 1% disodium EDTA solution. The pH was adjusted to 6.50 with 4 *M* sodium hydroxide and the resultant solution applied to a Bio-Rex 70 cation-exchange column prepared in a Bio-Rad plastic minicolumn. The columns were allowed to drain to waste, then 10 ml of distilled water were used to wash out the Sterilin containers onto the columns, which were again allowed to drain to waste. Large plastic tubes (Sarstedt) containing 2.5 g sodium chloride were placed under the columns and the methoxy compounds eluted with 6 ml of 4 *M* ammonium hydroxide. The tubes were capped and vortex-mixed to ensure saturation with sodium chloride, then 5 ml of ethyl acetate—acetone (2:1, v/v) were added followed by rotary mixing (Matburn) for 20 min.

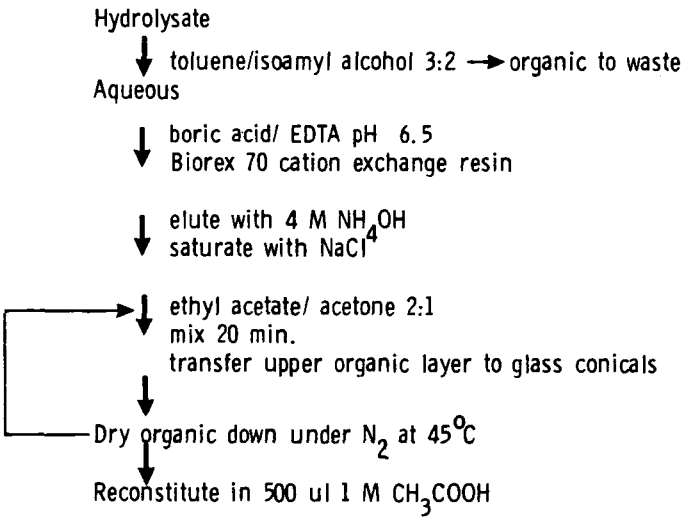

Fig. 2. Flow chart showing the extraction procedure.

The phases were separated by briefly centrifuging at 2050 *g*, the aqueous phase was retained and the upper organic layer was transferred to a conical glass tube. The extract was taken to dryness with nitrogen at 45° C. The aqueous phase was again extracted with 5 ml ethyl acetate—acetone and the extracts pooled for a final drying down, followed by reconstitution in 500 μl of 1 *M* acetic acid. An outline of the extraction procedure is given in Fig. 2.

Calculation

Measurement of 3-O-methyl isoprenaline was carried out by comparison of the peak height ratio of 3-O-methyl isoprenaline to 3-O-methyl isoetharine in the sample, to that obtained from authentic standards prepared in drug free plasma or urine, extracted and chromatographed in the same way. At least four such standards were run with each batch of samples.

RESULTS AND DISCUSSION

Chromatography

Our previous experiences in the separation of catecholamines and related compounds with citrate—phosphate buffers [29] led us to select a similar system, but of higher ionic strength in order to resolve the relatively less polar 3-O-methyl isoprenaline and 3-O-methyl isoetharine from each other. Preliminary experiments with authentic standards gave retention times of 6 and 14 min for 3-O-methyl isoprenaline and 3-O-methyl isoetharine, respectively. Hydrodynamic voltammograms of these two compounds were performed and as a result the optimum applied potential was found to be +0.80 V versus Ag/AgCl. Representative chromatograms of urine samples obtained with the method are shown in Fig. 3. No interfering peaks were observed at the retention time of the two analytes in the chromatograms of urines from *d*-isoprenaline-free subjects. The dopamine metabolite 3-methoxytyramine was present, but sufficiently well resolved from the 3-O-methyl isoprenaline peak to

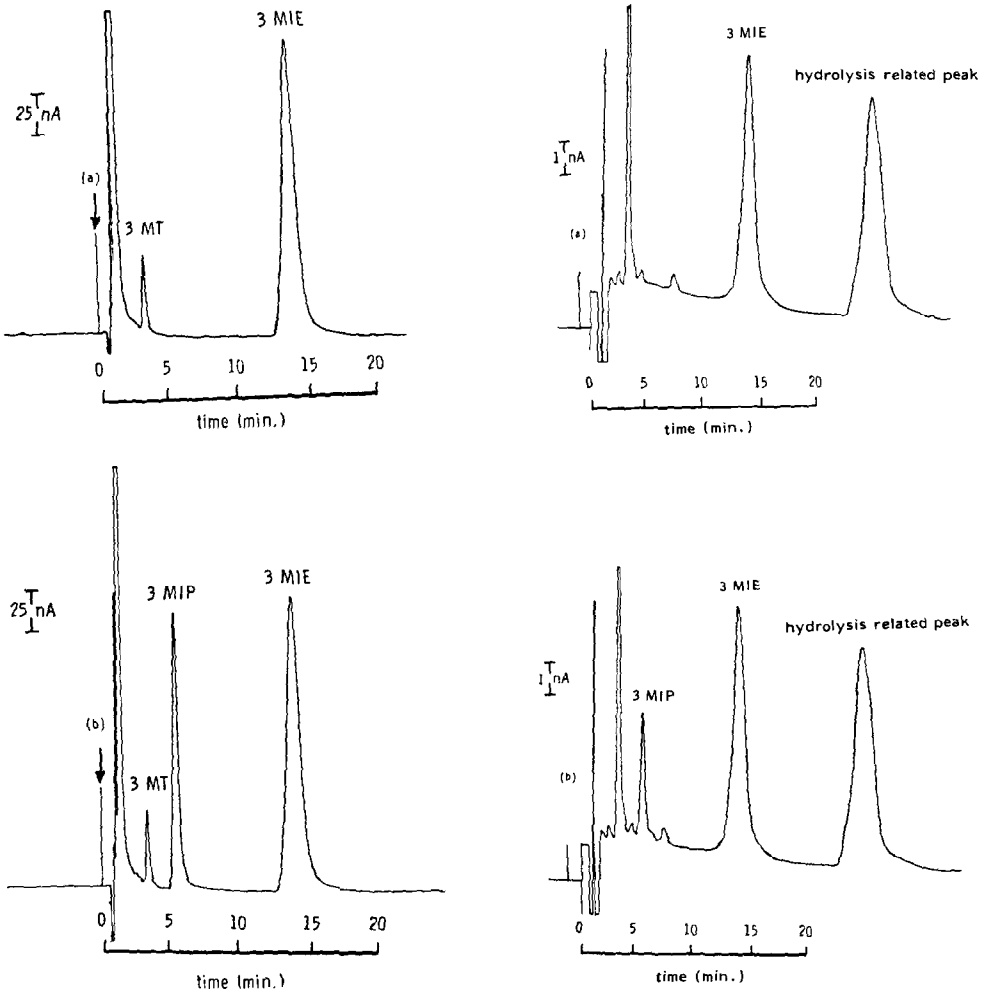

Fig. 3. Chromatograms of (a) 24-h urine from a drug-free subject; (b) 24-h urine from a subject after receiving 2 mg/kg *d*-isoprenaline-*d*-bitartrate. Chromatographic conditions as in text. Peaks: 3MT = 3-methoxytyramine; 3MIP = 3-O-methyl isoprenaline; 3MIE = 3-O-methyl isoetharine (internal standard).

Fig. 4. Plasma chromatograms from (a) drug-free subject; (b) 2.5 h after receiving an oral dose of *d*-isoprenaline (2 mg/kg). Peaks: 3MIE = 3-O-methyl isoetharine (internal standard); 3MIP = 3-O-methyl isoprenaline; hydrolysis related peak.

present no problems. Occasionally, broad, late eluting peaks were seen, towards the end of a batch of urine samples. Typical plasma sample chromatograms are displayed in Fig. 4. Again no interferences were seen in the chromatograms of drug-free plasma, although a large hydrolysis-related peak was present in all samples, with a retention time of 28 min — this extended the analysis time in plasma samples to 35 min.

Hydrolysis

Acidic hydrolysis is a rapid, inexpensive and effective procedure for the

deconjugation of catechol compounds. Being a chemical process it is not subject to the limitations of the enzymic methods using aryl sulphatase (EC 3.1.6.1) [30]. Our finding of quantitative conversion of the 3-O-methyl conjugates after 30 min at 100°C, is in close agreement with other published data [30—32].

Recovery

The absolute analytical recovery of authentic 3-O-methyl isoprenaline spiked into biological samples was 65% in urine (n = 5) and 68% in plasma (n = 5).

Quantitation

The linearity and precision of the measurement of 3-O-methyl isoprenaline by HPLC with amperometric detection, preceded by extraction from plasma or urine, were investigated by analysing samples spiked with known amounts of authentic standard. Calibration curves were constructed from the peak height ratio of 3-O-methyl isoprenaline:3-O-methyl isoetharine versus the concentration of 3-O-methyl isoprenaline, and were shown to be linear over the range 5 ng/ml to 20 μg/ml in plasma or urine. The limit of detection was 2 ng/ml at a signal-to-noise ratio of 2.0. Typical equations for calibration lines were $y = 0.18x + 0.1488$ ($r = 0.9958$) for plasma and $y = 0.0011x + 0.0343$ ($r = 0.9965$) for urine. Within-assay coefficient of variation of the assay was 4.5% (n = 15, $\overline{X}$ = 51.33 ng/ml, S.D. 2.3503) and the between-assay coefficient of variation 9.6% (n = 6, $\overline{X}$ = 51.83 ng/ml, S.D. = 4.9967).

Application to presystemic metabolism

The plasma concentration—time profiles for 3-O-methyl isoprenaline sulphate following the ingestion of 2 mg/kg *d*-isoprenaline-*d*-bitartrate are shown in Fig. 5. There is clearly a considerable inter-individual variation in response to this single oral dose, with peak concentrations of 3-O-methyl isoprenaline varying from only 48 up to 600 ng/ml and with the peak occurring from 2.0 to 5.0 h after the dose. It is possible to classify subjects into low and high areas under the plasma concentration—time curve (AUC). Thus subjects 3, 6 and 7 could be placed into a low AUC group and 1, 2, 4 and 5 into a high AUC group. The urinary excretion of 3-O-methyl isoprenaline sulphate ranged from 6.16 to 20.50 mg per 24 h, this representing between 4.7% and 13.5% of the oral dose given. The urine data are summarised in Table I and are in good agreement with earlier pharmacokinetic studies using tritium-labelled isoprenaline [33]. As expected there is a direct relationship between the plasma 3-O-methyl isoprenaline sulphate AUC and the percentage of the *d*-isoprenaline dose excreted as 3-O-methyl isoprenaline sulphate in the urine, i.e. with subjects in the low AUC group having a lower excretion of 3-O-methyl isoprenaline sulphate than their high AUC counterparts.

The individual variations seen may be due to variable absorption, metabolism or a mixture of the two. By extending the number of subjects and measuring both of the principal metabolites of isoprenaline at the same time, one should be able to investigate the possibility of an inverse relationship between 3-O-methyl isoprenaline sulphate and isoprenaline sulphate.

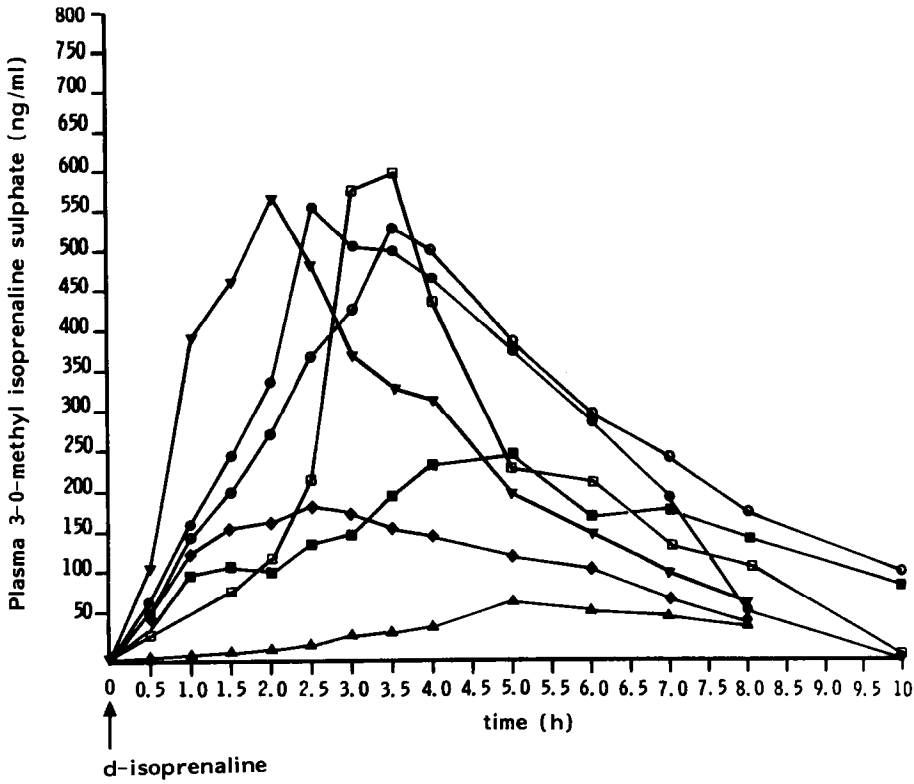

Fig. 5. Plasma concentration—time curves for 3-O-methyl isoprenaline sulphate in seven healthy volunteers. □, Subject 1; ●, subject 2; ■, subject 3; ▽, subject 4; ○, subject 5; ▲, subject 6; ◆, subject 7.

TABLE I

URINARY EXCRETION OF 3-O-METHYL ISOPRENALINE SULPHATE

Subject No.	Urinary excretion of 3-O-Methyl isoprenaline (mg per 24 h)	Percent of oral dose excreted as 3-O-methyl metabolite	Creatinine (g per 24 h)
1	19.80	13.5	1.99
2	20.50	12.3	1.89
3	15.70	9.2	2.17
4	18.00	13.4	1.74
5	16.54	11.2	2.01
6	6.16	4.7	1.54
7	11.80	7.8	1.86

CONCLUSIONS

A selective and sensitive HPLC method for the determination of 3-O-methyl isoprenaline sulphate in human plasma and urine is described. Cation exchange and solvent extraction are combined with reversed-phase ion-pairing HPLC and amperometric detection. It is now possible to study further the role of the gut

in the metabolic inactivation of catecholamines with particular interest in the widely postulated sulphate conjugation defect.

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