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DETERMINATION OF *d*-ISOPROTERENOL SULPHATE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH AMPEROMETRIC DETECTION

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SUMMARY

The application of reversed-phase high-performance liquid chromatography (HPLC) to the determination of isoproterenol sulphate in human plasma and urine was investigated. Sulphoconjugation of the inactive isomer of isoproterenol was chosen as an experimental model to study individual variations in the rate of sulphation of phenols in man. This approach allowed the ingestion of relatively large amounts of drug and detection of the conjugated material after acid hydrolysis, using alumina clean-up and HPLC with amperometric detection. This method was found to be rapid, sensitive, precise and suited to pharmacokinetic studies in man.

INTRODUCTION

The involvement of gut sulphoconjugation in the detoxification of phenols and catecholamines has been known for over a century [1]. Work by Richter [2] and Richter and MacIntosh [3] in the 1940s showed that in man 70% of an oral dose of adrenaline was excreted as the pharmacologically inactive suphate conjugate. More recently, the role of sulphate conjugation in pre-systemic elimination, has been recognised as a major pathway for the bio-transformation of phenolic drugs [4]; and this is of particular interest because of the limited capacity and susceptibility to competitive inhibition [5, 6].

Individual differences in the sulphate conjugation of paracetamol, shown by the skewed frequency distribution of the percentage of paracetamol sulphate excreted in the urine following oral dosing [7] and the report of two distinct forms of the sulphoconjugating enzyme, phenolsulphotransferase (EC 2.8.2.1,

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PST), in vitro [8, 9], lead us to speculate the possibility of an underlying polymorphism in gut sulphoconjugation.

Isoproterenol was chosen as a probe for sulphate conjugation of catecholamines in the gut, because it is a synthetic catecholamine available as the *d*-isomer, thus enabling a large oral dose to be given without pharmacological effect (*d*-isoproterenol has only 1% of the pharmacological potency of the *l*-isomer). It has the additional advantage of being both a good substrate for PST [10] and a poor substrate for the potentially competing enzyme monoamine oxidase [11]. Furthermore, earlier work in this department established that oral isoproterenol undergoes extensive pre-systemic metabolism in the gut wall with more than 80% of an oral dose of [^{3}H]isoproterenol being metabolised to isoproterenol sulphate [12—14].

The introduction of isoproterenol as a bronchodilator drug to treat asthma in the 1960s, led to the publication of several fluorimetric methods for its analysis [15, 16]. However, the definitive work on the pharmacology and metabolism of isoproterenol in man was achieved using radiolabelled isoproterenol, because at this time, scintillation counting of the tritium-labelled metabolites offered the most sensitive and specific approach [17]. Ultraviolet spectrophotometry [18] and gas—liquid chromatography with flame ionisation [19] have been applied to pharmaceutical dosage forms, but not to biological samples. More recently Kishimoto et al. [20] described a high-performance liquid chromatographic (HPLC) method involving post-column trihydroxyindole derivatization and fluorescence detection. This method utilised direct injection of urine and deproteinised plasma, but the combination of a 30-μm particle size cation-exchange column and the need for post-column derivatization lead to a long retention time for isoproterenol (24 min).

The reversed-phase ion-pairing HPLC procedure presented here employs N-methyldopamine as internal standard, a compound which is not normally administered or present as a metabolite in patients' plasma or urine. Isoproterenol sulphate is determined as isoproterenol following acid hydrolysis [21] and alumina batch extraction [22]. Following HPLC separation, the catechol groups are oxidised to orthoquinones using an amperometric detector, which also amplifies the current generated and displays this as a function of time on the chart recorder [23].

EXPERIMENTAL

Reagents and standards

N-Methyldopamine, *dl*-isoproterenol hydrochloride, 3′-phosphoadenosine-5′-phosphosulphate (PAPS) and tris(hydroxymethyl)aminomethane (Tris) were obtained from Sigma (Poole, U.K.). *d*-Isoproterenol-*d*-bitartrate (98% pure by thin-layer chromatography, TLC) was a gift from Sterling Winthrop (Surbiton, U.K.). AnalaR methanol purchased from May and Baker (Dagenham, U.K.) was found satisfactory. [^{35}S]PAPS, 1.9 Ci/mmol (NEG-010) was obtained from New England Nuclear (Boston, MA, U.S.A.) and *dl*-[7-^{3}H]isoproterenol hydrochloride, 12.6 Ci/mmol (TRK 295) from Amersham International (Amersham, U.K.). All other chemicals were of analytical reagent grade and obtained from BDH chemicals (Enfield, U.K.). The activated alumina (Brockmann grade 1,

active, neutral) was prepared as previously described [24]. The internal standard, N-methyldopamine, was prepared as a 1 μg/ml solution in 0.1 *M* hydrochloric acid and stored at 4°C. A stock standard of 6 mg/ml *d*-isoproterenol-*d*-bitartrate was similarly prepared. Standard curves of subdiluted stock standard were prepared in isoproterenol-free urine and plasma.

Synthesis of isoproterenol sulphate

Since isoproterenol sulphate was not commercially available, it was synthesized from isoproterenol and PAPS in a reaction catalysed by partially purified rat liver PST. The isolation of PST was essentially that of Gregory and Lipmann [25] and the preparation of isoproterenol sulphate was based on the method of Foldes and Meek [26]. Initially, [^{35}S] PAPS was used in the reaction to optimise the production and separation of the sulphate conjugate. Once achieved this was replaced by [^{3}H] isoproterenol and unlabelled PAPS, in order to produce [^{3}H] isoproterenol sulphate, needed for hydrolysis recovery studies. The reaction was carried out with 1 m*M* *dl*-isoproterenol hydrochloride (containing 252 mCi of *dl*-[^{3}H] isoproterenol hydrochloride) at pH 8.0 and the product was separated from the starting material by silica gel TLC using butanol—0.880 ammonia—ethyl acetate (60:20:40) as the solvent system. Only two radioactive peaks were detected: the starting material, [^{3}H] isoproterenol with an R_F of 0.71 and the product presumed to be [^{3}H] isoproterenol sulphate, having an R_F of 0.32. This latter TLC band contained 68% of the radioactivity of the starting material. Acidic hydrolysis of the product gave a single radioactive peak with an R_F identical to that of [^{3}H] isoproterenol and all of the radioactivity of the conjugate was recovered. The tritium-labelled conjugate was not extracted by activated alumina, this providing additional evidence that one of the phenolic hydroxyl groups is replaced by sulphate. In combination, these findings provide good evidence for the successful synthesis Downers Grove, IL, U.S.A.).

Equipment

The liquid chromatograph comprised an Altex 100A solvent delivery pump, an Altex 210 injection valve fitted with a 100-μl sample 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 detection system comprised a Model LC4 amperometric detector fitted with a TL5 glassy carbon electrode assembly (Bioanalytical Systems, West Lafayette, IN, U.S.A.). The column, amperometric detector and electrode were enclosed in a Faraday cage of aluminium, itself earthed to the Servoscribe RE 541.20 chart recorder (Smiths Industries, London, U.K.). For TLC work, LK 5 DF silica gel TLC plates (Whatman, Maidstone, U.K.) were used together with a TLC Chromatank (Shandon Southern, Runcorn, U.K.). Radiochromatograms were obtained using a Packard Model 7201 scanner (Packard Instruments, Downers Grove, IL, U.S.A.).

Chromatography

The mobile phase consisted of a citrate—phosphate (McIlvaine) buffer adjusted to pH 6.0, containing 3% v/v methanol and the disodium salt of EDTA

at a final concentration of 2 m*M*. The buffer was composed of 300 ml of 0.1 *M* citric acid and 150 ml of 0.1 *M* disodium hydrogen phosphate per l of glass-distilled water. Solvent was filtered through a 0.5-μm Gf/f glass microfibre filter (Whatman) and helium-degassed (BOC Special Gases, London, U.K.) prior to use. The HPLC system was maintained in continuous use at a flow-rate of 0.1 ml/min and the flow increased to 1.0 ml/min prior to sample work-up and injection. The amperometric detector was typically used at a sensitivity of 10 nA full scale and an applied potential difference of +0.50 V vs. Ag/AgCl reference electrode.

Optimisation of chromatography

In Moyer and Jiang's paper [27] the optimal mobile phase for analysis of catecholamines by HPLC with amperometric detection was established as being in the pH range 5.0—6.5, with an ionic strength in the region of 0.07 *M* (phosphate ion) and with an ion-pair such as heptanesulphonate at 5.0 m*M*. Our previous experiences with reversed-phase ion-pairing HPLC systems for the determination of plasma and urinary catecholamines [28—31] suggested that a reduction in both ion-pair concentration and methanol content of the mobile phase, would be necessary to achieve a practical retention time for the less polar synthetic catecholamines isoproterenol and N-methyldopamine.

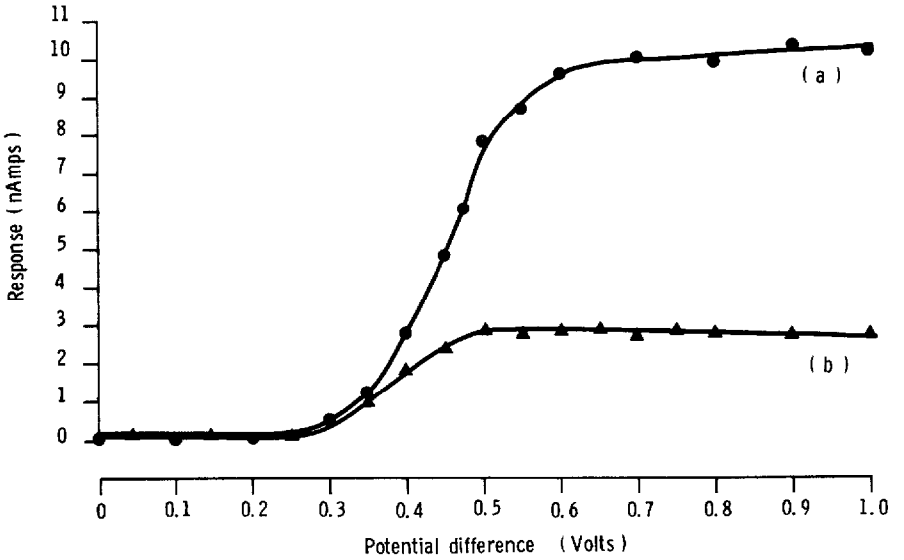

Fig. 1. Hydrodynamic voltammograms for isoproterenol (a) and N-methyldopamine (b) in mobile phase pumped at a flow-rate of 1.0 ml/min.

Hydrodynamic voltammograms for isoproterenol and N-methyldopamine were determined over the range 0—1.0 V vs. Ag/AgCl reference electrode, in the mobile phase described (Fig. 1). An applied electrode potential of +0.50 V was chosen (as in Moyer and Jiang's paper [27]) as this provided sufficient sensitivity for the measurement of plasma and urinary isoproterenol sulphate, but with minimal interference from solvent oxidation and electrical noise.

Sample handling and investigation protocol

Four healthy volunteers (mean age 27.5 years) were asked to avoid headache and cold medicines containing paracetamol or ascorbic acid for 12 h prior to dosing and to avoid eating breakfast, but they were allowed to eat lunch 3 h after taking the dose. On the day of the investigation subjects emptied their bladders at 10 a.m., a butterfly cannula was inserted into a forearm vein and kept patent using heparinised saline. A baseline blood sample (10 ml) was drawn into a lithium heparin tube and the oral dose of *d*-isoproterenol-*d*-bitartrate (1 mg/kg) was given in 330 ml of a proprietary drink, which contained no ascorbic acid [32]. A 24-h urine collection was started and further blood samples were taken at 30-min intervals up to 6 h. A second 24-h urine was started as the first collection finished at 10 a.m. the next day. 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.

The blood samples were centrifuged at 2250 *g* for 15 min at 4°C and the separated plasma stored at −70°C until assayed.

Hydrolysis of isoproterenol sulphate

The unimolecular acid-catalysed hydrolysis of isoproterenol sulphate proved to be simple, quick, inexpensive and quantitative with 98% recovery of [^{3}H]isoproterenol sulphate spiked into plasma and urine. Sulphatase (H-1, from *Helix pomatia*) catalysed hydrolysis of [^{3}H]isoproterenol sulphate was also quantitative from urine but less than 20% efficient with plasma samples. The method of acid hydrolysis involved mixing 2 ml of plasma or urine with 2 ml of 1.0 *M* hydrochloric acid and placing the mixture in a boiling water bath for 30 min. Portions of hydrolysed plasma (2 ml) were transferred into extraction tubes while still warm to avoid gel formation on cooling. Hydrolysed urines were diluted 1:50 and 2-ml aliquots of the dilution taken for extraction.

Validation of sulphoconjugate

Phenolic compounds can be conjugated with glucuronic acid, sulphate or both. Previous work on isoproterenol has indicated very little glucuronide conjugation [17]. Evidence that the compound was a sulphate and not a glucuronide was obtained using sulphatase-catalysed hydrolysis in the presence of the glucuronidase inhibitor saccharic acid 1:4 lactone [33]. This was found to be without effect on the sulphatase-catalysed hydrolysis of the conjugate at concentrations up to 10 mg/ml, indicating that sulphate is the only appreciable conjugate of isoproterenol.

Extraction of isoproterenol

The hydrolysates (2 ml) were placed into conical polystyrene tubes (Sarstedt, Leicester, U.K.) containing 60 mg of activated alumina, 1 ml of 0.1 m*M* Na_2EDTA with 1 m*M* hydrochloric acid and 100 μl of 1 μg/ml N-methyldopamine (internal standard). The pH was adjusted to 8.6 by the addition of 1 ml of 3 *M* Tris—HCl buffer and the tubes were mixed on a Spiramix roller mixer (Denley, Billinghurst, U.K.) for 15 min. The alumina was allowed to settle and the supernatants were aspirated at the sink followed by three washes

with distilled water (pH 7.0). The final aspiration was taken to dryness and the catecholamines were eluted in 200 μl of 0.1 *M* orthophosphoric acid by roller mixing for 2 min. Fines of alumina were centrifuged down (2200 *g*, 4°C, 5 min) and the supernatants finally transferred into Eppendorf tubes (1.5 ml). The supernatants were stored at −20°C for up to one week, if not chromatographed on the same day.

Injection volumes

In order to achieve on-scale peaks for all samples, 50-μl aliquots of hydrolysed urine (diluted 1:100 overall) or hydrolysed plasma (diluted 1:2 overall) were injected into the HPLC system. For unhydrolysed urines 25-μl and for unhydrolysed plasma 100-μl portions of supernatant were injected.

Quantitation

Measurement of *d*-isoproterenol was by comparison of the peak height ratio of isoproterenol to N-methyldopamine 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 five such standards were run with each batch of samples.

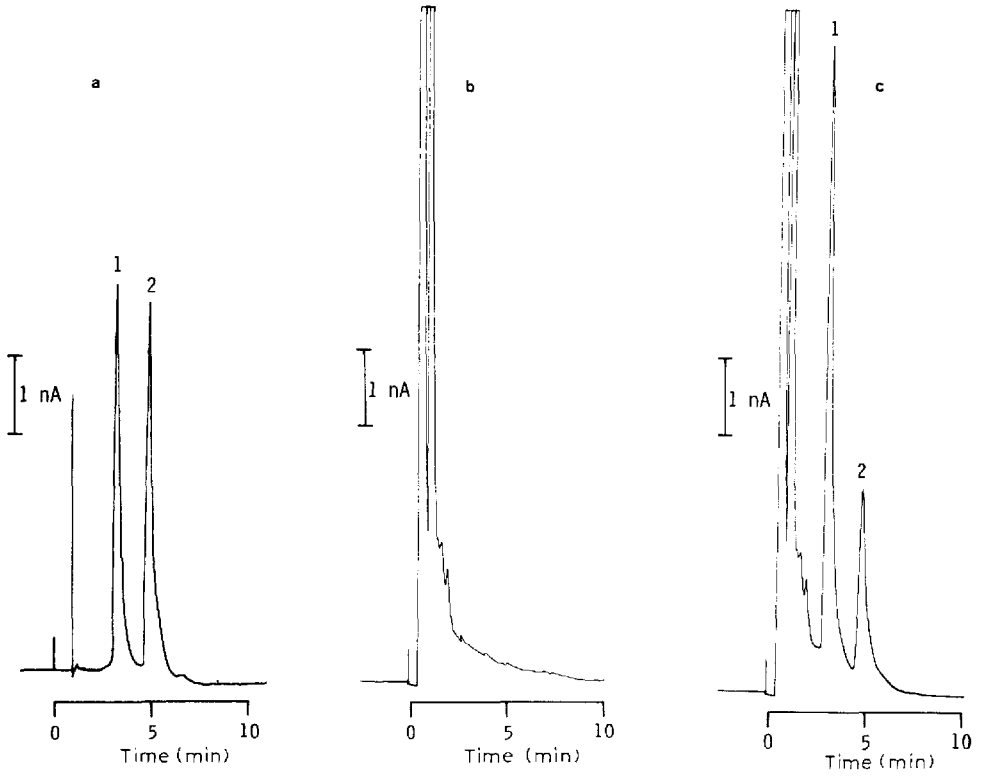

Fig. 2. Chromatograms showing HPLC—amperometric assay of plasma *d*-isoproterenol. (a) Standard mixture containing 100 ng each of N-methyldopamine (1) and *d*-isoproterenol (2); (b) plasma from a drug-free subject; (c) plasma from a subject 30-min post-dose of 1 mg/kg *d*-isoproterenol. Concentration of free isoproterenol is 20 ng/ml.

RESULTS AND DISCUSSION

Chromatography

Resolution and sensitivity of the chromatographic system were determined for each assay by injection of a reference solution containing *d*-isoproterenol and N-methyldopamine. A typical chromatogram obtained from such an injection is shown in Fig. 2a. Chromatograms produced by extracted plasma samples are shown in Fig. 2b and c and Fig. 3, while representative chromatograms obtained from extracted 24-h urine collections are given in Fig. 4.

Validation of the *d*-isoproterenol peak in plasma and urine samples from dosed subjects was obtained by the finding of co-chromatography with authentic *d*-isoproterenol.

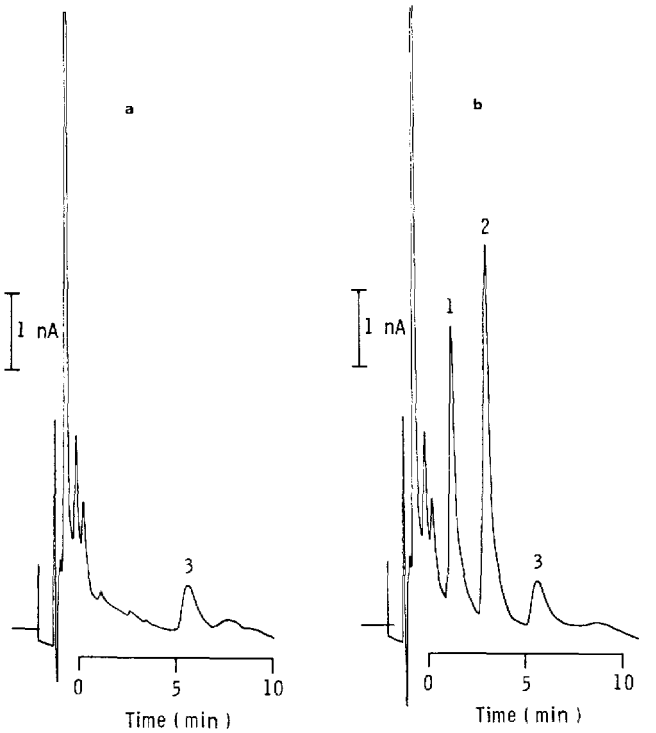

Fig. 3. Chromatograms showing HPLC—amperometric assay of *d*-isoproterenol in hydrolysed plasma. (a) Plasma from a drug-free subject; (b) plasma from a subject 30-min post-dose of 1 mg/kg *d*-isoproterenol. Concentration of isoproterenol sulphate is 196 ng/ml. For peaks 1 and 2 see Fig. 2. Peak 3 is an unassigned peak only present in hydrolysed samples.

Selectivity

The possibility of endogenous interferences in plasma or urine was investigated at the retention times of *d*-isoproterenol and N-methyldopamine (Table I). No interference was seen in the chromatograms of samples extracted without added internal standard or in samples from isoproterenol-free subjects. However, in the chromatograms of acid-hydrolysed samples an extra peak (labelled 3 in the chromatograms) was seen after the isoproterenol peak, with a retention time of 7.6 min. This peak was not related to the dose of iso-

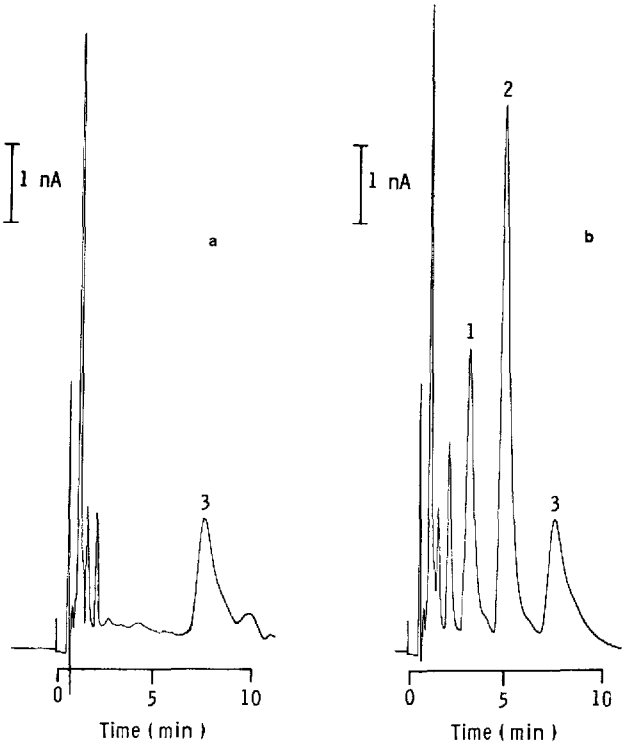

Fig. 4. Chromatograms obtained from extracted hydrolysed urines. (a) 24-h urine from a drug-free subject; (b) 24-h urine from a subject who received 1 mg/kg *d*-isoproterenol at the start of the 24-h period. Peaks: (1) N-methyldopamine; (2) *d*-isoproterenol; (3) unassigned hydrolysis peak.

TABLE I

RETENTION TIMES OF CATECHOL COMPOUNDS

For conditions, see text.

Compound	Retention time (min)
3,4-Dihydroxymandelic acid (DHMA)	0.7
3,4-Dihydroxyphenylacetic acid (DOPAC)	0.9
3,4-Dihydroxyphenylalanine (DOPA)	1.0
3,4-Dihydroxyphenylglycol (DHPG)	1.4
Dopamine	1.8
N-Methyldopamine	3.1
3-Methoxy-4-hydroxyphenylglycol (MHPG)	4.2
Isoproterenol	5.3
Unassigned hydrolysis-related peak	7.6
3-O-Methylisoproterenol	15.8

proterenol given and was present in acid-hydrolysed samples from drug-free subjects. It is therefore concluded that the peak derives from an endogenous acid labile conjugate. Since it it is well resolved from N-methyldopamine and isoproterenol the peak does not interfere with the measurement of

isoproterenol, other than to increase analysis time (in hydrolysed samples) to 10 min, in order to ensure its elution before the next sample is injected.

Precision

The intra-assay precision of the method was determined by replicate analysis of a drug-free plasma and urine pool spiked with authentic *d*-isoproterenol. This gave a coefficient of variation of 2.6% for plasma ($\overline{X}$ = 632.6 ng/ml, S.D. = 16.286, n = 15) and 3.1% for urine ($\overline{X}$ = 50.26 mg per 24 h, S.D. = 1.541, n = 15).

The inter-assay precision was measured by analysis of the same spiked samples, one in each of six separate assays. This gave a coefficient of variation of 4.6% for plasma ($\overline{X}$ = 617.0 ng/ml, S.D. = 28.239, n = 6) and of 7.1% for urine ($\overline{X}$ = 49.48 mg per 24 h, S.D. = 3.54, n = 6).

Calibration

Standard curves were found to be linear over the range 1 ng/ml to 50 μg/ml *d*-isoproterenol, with a correlation coefficient of 0.9996 and linear-regression equation of $y = 7.762x + 0.177$.

Limit of detection

With the amperometric detector at a sensitivity of 10 nA full scale and an applied potential of +0.50 V vs. Ag/AgCl reference electrode, the limit of detection was 0.5 ng/ml at a signal-to-noise ratio of 2.0. This detection limit could be extended into the picogram range by increasing the detector amplification.

Recovery

The absolute analytical recovery of *d*-isoproterenol and N-methyldopamine for human plasma and urine was estimated by comparing the peak heights obtained from the injection of known quantitites of the compounds, with peak heights obtained from the injection of extracts of plasma and urine samples spiked with the analytes. This gave values of 71% for *d*-isoproterenol and 68% for N-methyldopamine (n = 8).

Pharmacokinetics

Practical application of the proposed method was tested in human plasma and urine samples, collected from subjects following the oral ingestion of *d*-isoproterenol. A limited dose/response (d/r) curve was undertaken in one subject (range 0.5—1.5 mg/kg) and plasma isoproterenol sulphate determined by HPLC with amperometric detection (Fig. 5). The peak plasma isoproterenol sulphate concentration occurred between 1.5 and 2.0 h post-dose and increased in a dose-dependent fashion. Plasma isoproterenol sulphate—time curves in four subjects, following the oral dose of 1 mg/kg *d*-isoproterenol-*d*-bitartrate are shown in Fig. 6.

The area under the plasma concentration—time curve (AUC) was calculated assuming monoexponential kinetics and applying a computer program using the trapezoidal rule. Although these kinetics are not proven, the AUC values thus calculated should be a good approximation, even if there is a longer

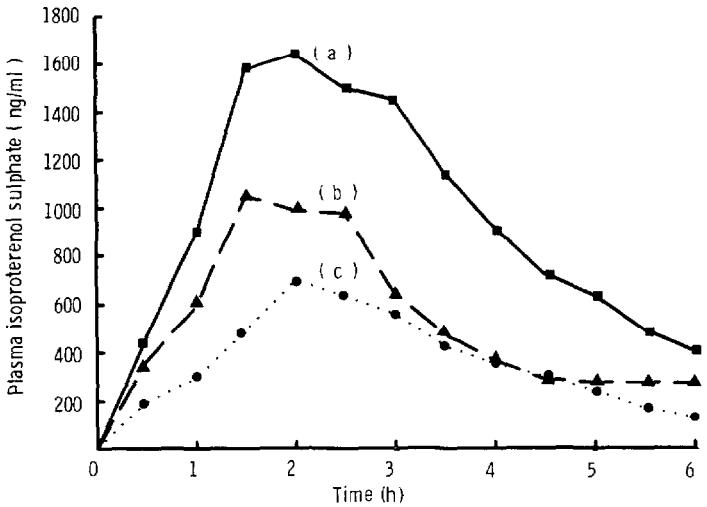

Fig. 5. Dose/response curves of plasma isoproterenol sulphate following (a) 1.5 mg/kg; (b) 1.0 mg/kg; and (c) 0.5 mg/kg *d*-isoproterenol-*d*-bitartrate as an oral dose.

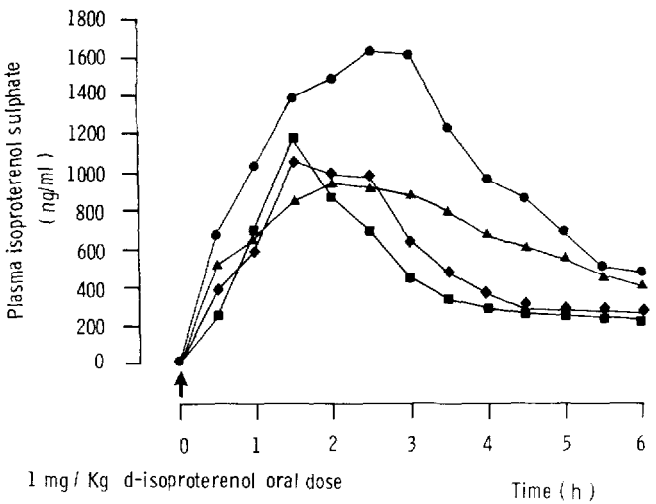

Fig. 6. Plasma isoproterenol sulphate—time curves in four healthy subjects after an oral dose of 1 mg/kg *d*-isoproterenol-*d*-bitartrate.

terminal phase in the elimination. The renal clearances were estimated from the venous plasma AUC values and take no account of possible arterio—venous concentration differences [34]. A results summary showing the urinary values together with derived pharmacokinetic parameters is given in Table II.

CONCLUSIONS

The determination of isoproterenol sulphate as isoproterenol by HPLC with amperometric detection, following acid hydrolysis of the conjugate and alumina batch extraction, gave excellent reproducibility and a short chromatography time of 6 min per sample.

TABLE II

PHARMACOKINETIC PARAMETERS FOLLOWING A 1 mg/kg DOSE OF *d*-ISOPROTERENOL-*d*-BITARTRATE IN HEALTHY HUMAN SUBJECTS

Subject No.	Oral dose excreted as isoproterenol sulphate (%)	Plasma isoproterenol sulphate AUC* (μg/ml min)	Renal clearance of isoproterenol sulphate** (ml/min)
1	57	243	171
2	41	169	154
3	76	242	242
4	99	368	166

*Calculated by the trapezoidal rule.
**Calculated using venous blood samples.

The single subject d/r curve shows that 1 mg/kg *d*-isoproterenol does not saturate the gut PST and is therefore suitable for studies of genetic polymorphism in PST. Plasma isoproterenol sulphate AUC values were proportional to dose and there was no significant change in the percentage of the dose excreted as the sulphoconjugate, as the dose increased from 0.5 to 1.5 mg/kg.

In four healthy volunteers considerable variation in the percentage of the 1 mg/kg dose, excreted as isoproterenol sulphate, was observed. However, very little free isoproterenol was found in the urine and it did not show the same degree of variation as the sulphoconjugate. Some evidence for the renal secretion of *d*-isoproterenol sulphate was obtained from an estimation of its renal plasma clearance, this being greater than the glomerular filtration rate in the period 0—6 h post-dose. The slow decline in plasma levels between 4 and 6 h post-dose may be attributed to a longer terminal phase and this could be investigated by collecting blood samples for longer periods of time. Prolongation of oral absorption may also account for the observed data. Further characterisation of the pharmacokinetics of *d*-isoproterenol sulphate thus requires the collection of more blood samples after 6 h and the investigation of a greater number of volunteers.

These preliminary results confirm that conjugation with sulphate accounts for a major proportion of the pre-systemic elimination of oral *d*-isoproterenol. Excretion of free isoproterenol did not account for a high percentage of the dose. It seems likely that low sulphate conjugation may be accompanied by increased O-methylation, and in future studies we plan to measure the plasma and urinary isoproterenol sulphate/3-O-methylisoproterenol ratio.

The HPLC amperometric method described in this paper has been shown to be suitable for studies on the conjugation of isoproterenol with sulphate. Since isoproterenol is almost exclusively sulphate-conjugated, it should provide a handle on the study of inter-individual variation in sulphation of phenols by man, in combination with the measurement of PAPS and sulphate. These studies will complement those on paracetamol — a drug which is conjugated equally with sulphate and glucuronic acid and which can also be measured by HPLC with amperometric detection [35].

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