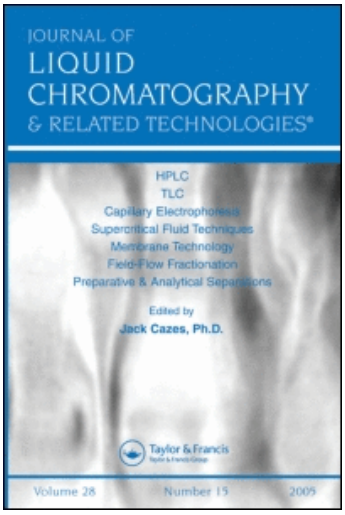

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THE DEVELOPMENT OF A HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY METHOD TO DETERMINE PYRUVATE GENERATED DURING THE C-S LYSIS OF CYSTEINE CONJUGATED XENOBIOTICS

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ABSTRACT

It is now well recognized that cysteine conjugates are sometimes responsible for the mutagenic and cytotoxic consequences which occur following the ingestion of halocarbons. These toxic reactions arise from the generation of reactive metabolites *via* the cleavage of the C-S bond in cysteine conjugates. The enzymes which facilitate this reaction on cysteinyl conjugate metabolites are now known as C-S lyases. The products of the C-S lyase catalysis of cysteine conjugates are a reactive thiol, pyruvic acid and ammonia. Here we report the development of a sensitive fluorimetric hplc assay for pyruvic acid using the derivatising agent 1,2-phenylenediamine. It was found that using this assay CSL activity could be detected in a porcine C-S lyase preparation. It was also observed that this assay was approximately ten-fold more sensitive than the existing spectrophotometric assay.

INTRODUCTION

Many xenobiotics undergo conjugation reactions with glutathione with the glutathione conjugates then frequently undergoing further metabolism by any of a large number of pathways to afford various highly polar and water-soluble metabolites. These metabolic

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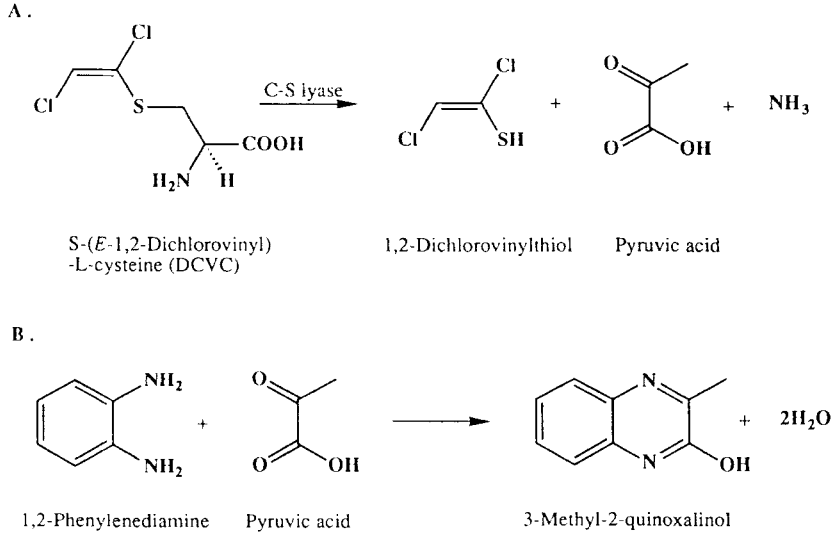

Figure 1. (A) C-S lyase catalysed cleavage of S-(E-1,2-dichlorovinyl)-L-cysteine. (B) Derivatisation reaction of 1,2-phenylenediamine with pyruvic acid.

products, the most important of which in mammals are the mercapturic acids (S-substituted N-acetylcysteines) are then excreted in urine or bile. The pathway leading to the formation of mercapturic acids has been generally regarded, until recently, as detoxifying. However, it is now recognized that the cysteine conjugate intermediate in the above mercapturate biosynthesis is sometimes responsible for the mutagenic and cytotoxic consequences which occur following the ingestion of halocarbons [1, 2]. These toxic reactions arise from the generation of reactive metabolites *via* the cleavage of the C-S bond in cysteine conjugates; the products of such bond lysis are a reactive thiol, pyruvic acid and ammonia (Fig.1A).

Enzymes capable of C-S bond lysis have been identified and purified from various human tissues [3, 4], and this has led to concerns over the impact of such a family of enzymes on the health of workers exposed to halogenated hydrocarbons which are metabolised in man via glutathione conjugation. Various assay techniques have been utilised in order to study the biochemistry of C-S lyase enzymes. In theory, it is possible to determine the levels of any of the products of C-S lysis. However, the development of a sensitive assay which is both accurate and precise, and has a high degree of specificity, has proven difficult.

Here we report the development of a sensitive assay for pyruvate using the derivatising agent 1,2-phenylenediamine and employing high performance liquid chromatography with fluorescence detection.

MATERIALS AND METHODS

Materials

S-(*E*-1,2-Dichlorovinyl)-L-cysteine) (DCVC) was synthesised in our laboratories by the method of McKinney *et al.* [5]. All other reagents used in this study were purchased commercially (Sigma Chemical Co., Poole, UK; Fluka, Glossop, UK) and were of at least analytical grade.

Enzyme incubations

C-S lyase enzyme was prepared from a porcine source [6]. All enzyme incubation solutions were prepared in aqueous phosphate buffer pH 7.4 and pre-incubated at 37°C. DCVC solution (10mM, 0.3ml) and enzyme solution (1mg protein/ml, 0.1ml) were placed in an tube and incubated at 37°C for 30min. Controls were prepared by substituting each solution with buffer only. Perchloric acid (18% aqueous solution, 0.1ml) was then added to the incubation mixture to terminate the reaction by precipitating the enzyme and the resulting suspension was then centrifuged (10min, 10,000x*g*).

HPLC apparatus

The HPLC system consisted of a pump equipped with a manometric module (Gilson Models 302 and 802C respectively, Gilson International, Villiers-le-Bel, France), a Rheodyne 7125 injection valve (Rheodyne, Cotati, CA, USA), a fluorescence spectrophotometer (Model PE3000, Perkin-Elmer, Beaconsfield, UK) equipped with an HPLC flow cell and a Spectra-Physics data integrator (Model SP4290, Spectra-Physics, San Jose, CA, USA). The analytical column used for the chromatography was a Spherisorb 5-μm ODS-1 column (25cm x 4.6 mm I.D., Hichrom, Reading, UK).

Derivatisation and HPLC

To deproteinised sample (0.5ml) or working aqueous pyruvate standard solution (0.1ml), 1,2-phenylenediamine solution in distilled water (0.13% w/v, 1.5ml) and aqueous 2-mercaptoethanol solution (1%, 5μl) were added, followed by distilled water to give a final volume of 3ml. These mixtures were heated in an 80°C water bath (60min), then cooled to room temperature in ice prior to the addition of anhydrous sodium sulphate (0.5g). The

derivatised sample was then extracted with ethyl acetate (3x3ml) and tumbled for 10min on a rotary mixer. The 9ml combined organic phase was dried over anhydrous sodium sulphate (0.5g) and evaporated to dryness under a stream of nitrogen. The dry residue was redissolved in methanol (0.2ml) and an aliquot (20µl) was injected onto the HPLC column. The column was eluted isocratically with methanol: water (6:4 v/v) at a flow rate of 1ml/min. The optimum excitation wavelength was determined as 350nm with emission at 410nm.

RESULTS

1,2-Phenylenediamine is a derivatisation reagent specific for α-keto acids [7], and it is likely that the derivatised product formed with pyruvic acid as the analyte is 3-methyl-2-quinoxalinol (Fig.1B). Whilst 3-methyl-2-quinoxalinol absorbs in the UV region, in order to maximise sensitivity, fluorescence detection was employed in this assay. The optimum excitation wavelength was found to be 333nm with emission at 400nm. The assay was clearly able to detect pyruvate in both standard solutions and in enzyme incubations. This can be observed by an examination of the chromatograms in Fig.2 which show a sample chromatogram obtained after derivatisation of a pyruvate standard solution and a chromatogram obtained after derivatisation of an enzyme incubation containing C-S lyase enzyme and DCVC. DCVC is cleaved by C-S lyase enzymes to yield a thiol, pyruvate and ammonia (Fig.1A). The pyruvate produced may be easily detected using the assay developed and reported here. The third chromatogram shown in Fig.2 is that of sample of 3-methyl-2-quinoxalinol, which has a similar retention time to that of the derivatised pyruvate sample. The retention time of both derivatised pyruvate and 2-quinoxalinol was found to be 4.50±0.03min.

A standard curve was prepared using a pyruvic acid stock standard solution (30mg/dl, 2.73mM). A linear function was fitted to each data set using linear regression analysis, the R^2 value being greater than 0.996. Linearity was observed over a concentration range of 0 to 12µM, being maintained up to a value 150µM. A calibration curve is shown in Fig.3. A standard curve was also prepared using commercially prepared 3-methyl-2-quinoxalinol which was also shown to be linear over this range. The assay was also shown to be precise with inter- and intra-day variability below 10% at concentrations greater than 0.3µM pyruvate. The assay method developed here was also compared with an established LDH biochemical spectrophotometric assay for pyruvate established within our laboratories and the results of this comparison are shown in Table 1.

These data demonstrate that the the minimum detectable concentration of pyruvate (i.e. where the t value exceeds the critical value) was found to be approximately 0.2µM. This is some ten-fold more sensitive than the LDH spectrophotometric assay used for comparison.

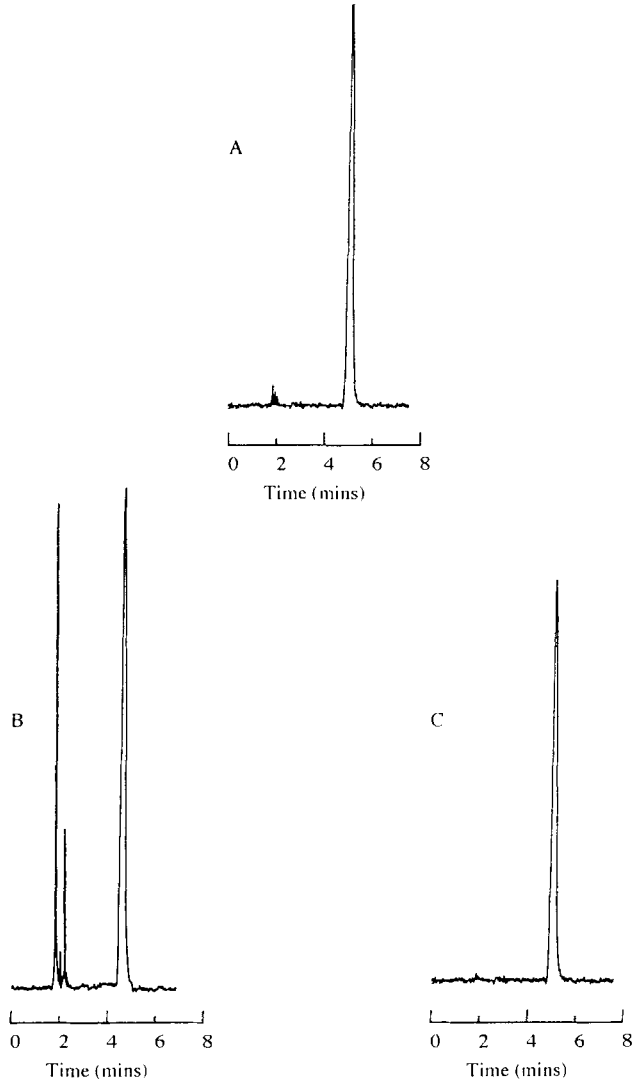

Figure 2 (A) Chromatogram obtained after derivatisation of pyruvic acid in solution.
(B) Chromatogram obtained after derivatisation of an enzyme incubation containing DCVC.
(C) Chromatogram of 3-methyl-2-quinoxalinol.

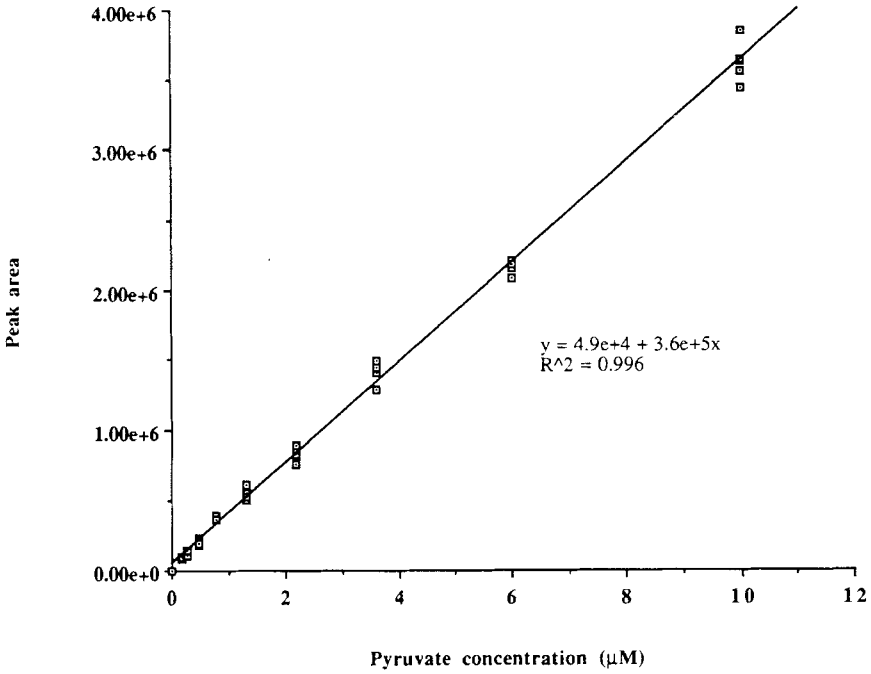

Figure 3 Calibration curve for pyruvic acid

TABLE 1

Comparison of Assay Variability for the HPLC Fluorescence Assay for Pyruvate and the LDH Spectrophotometric Assay.

LDH SPECTROPHOTOMETRIC ASSAY		HPLC FLUORESCENCE ASSAY	
Pyruvate (μM)	t value*	Pyruvate (μM)	t value
70.47	0.8	10	0.65
50.36	1.8	6	2.00
40.24	0.9	3.6	2.26
30.22	0.2	2.16	0.65
20.15	2.7	1.296	1.91
10.05	0.5	0.777	4.30
0.00	0.3	0.467	1.77
		0.280	2.73
* t_{crit}=4.3 at p 0.05		0.168	5.83

DISCUSSION

Enzymes which are responsible for catalysing the lysis of C-S bonds are known as C-S lyases and, when such enzymes are incubated with cysteine conjugates will yield a thiol moiety, pyruvic acid and ammonia in stoichiometric amounts. In order to study C-S lyase enzymes extracted from biological material a sensitive assay method for either the substrate or one of the products is required. A specific assay for the substrate is of lesser interest since such an assay would require modification for each compound under assay and would preclude structure-activity relationship studies.

The isolation and characterisation of the thiol containing moiety would give the most relevant index of C-S lyase mediated activity and hence, an indication of the toxic potential of a given substrate, since it is the thiol containing moiety which is known to cause toxicity. However, due to the highly reactive nature of these compounds their presence in solution is transient rendering conventional chromatography unsuitable. Dohn and Anders reported an assay for the determination of 2-mercaptobenzothiazole (MBT), the cleavage product of 2-benzothiazolyl-L-cysteine (BTC) [8]. However, the validity of this method was questioned by McFarlane and coworkers [9]. These workers failed to detect rat renal C-S lyase activity with 2-benzothiazolyl-L-cysteine as a substrate, when using pyruvate assay methods. Suzuki and coworkers have reported a method for the detection of 4-bromothiophenol (BTP) using high performance liquid chromatography (HPLC) [10]. BTP is produced by the action of C-S lyase on 4-bromophenyl-L-cysteine [10]. It has also been demonstrated that BTP and the internal standard, 4-fluorothiophenol are unstable under the assay conditions reported [11]. The methods of thiol detection so far reported are specific for a given chromophoric substrate.

Thiol groups can be readily oxidised forming disulphides (thioethers). The aromatic disulphide reagents 5,5'-dithiobis(2-nitrobenzoic acid (DTNB), 2,2'-dithiodipyridine, and 4,4'-dithiodipyridine are the reagents of choice for the detection of thiol groups. These reagents oxidise free thiol groups facilitating the release of an intensely chromophoric aromatic thiol. However, it has been shown in our laboratories that different thiols give varying chromophoric yields over the same concentration range[11]. This is a considerable disadvantage since frequently the thiols of interest in C-S lyase studies are not available as standards. Also, being non-specific, these reagents cannot be used in the presence of dithiothreitol or with crude tissue preparations [12]. Other possible reagents for the detection of thiols are discussed by Jocelyn [13].

Ammonia levels have previously been determined using a method adapted from that of Green and Odum, Evans and coworkers and Ishihara *et al.*[2, 14, 15]. In the presence of NADH, L-glutamate dehydrogenase catalyses the formation of L-glutamate from 2-oxoglutarate and ammonia. Again the consumption of NADH and the concomitant fall in

absorbance at 340nm can be determined. Pyruvate levels have been determined previously using various techniques. A method involving the formation of the dinitrophenylhydrazine derivative of pyruvate has been reported [16]. However, the hydrazine is non-specific for pyruvate, reacting with all compounds with a carbonyl functionality, and as such large experimental errors are incurred when using crude enzyme preparations. An enzyme-coupled spectrophotometric assay has been used by Green and Odum [2], McFarlane and coworkers [9], and our group [3,4,6]. This assay was adapted from that of Gutmann and Wahlefeld [17]. The reduction of pyruvate to form lactate, in the presence of the reduced form of nicotinamide adenine dinucleotide (NADH), is catalysed by the enzyme lactate dehydrogenase (LDH). The pyruvate consumed is proportional to the NADH oxidised which can be monitored due to the concomitant fall in absorption at 340nm. The use of the LDH ensures a higher degree of specificity than can be afforded using the hydrazine reagent.

The pyruvate assay developed here is specific and is at least ten-fold more sensitive than the pyruvate assay previously used. We believe that the HPLC assay reported here will greatly assist in studies involving C-S lyase enzymes and will thus aid in the prediction of mammalian toxicity observed after exposure to halogenated chlorofluorocarbons.

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