Journal of Chromatography, 641 (1993) 249–255
Elsevier Science Publishers B.V., Amsterdam

CHROM. 25 129

Isomeric separation of Beraprost sodium using an α_1-acid glycoprotein column

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(First received March 9th, 1993; revised manuscript received March 30th, 1993)

ABSTRACT

The separation of the four stereoisomers present in Beraprost sodium, a prostacyclin analogue, has been accomplished using an α_1-acid glycoprotein stationary phase (Chiral AGP column). The stereoisomers are baseline resolved with a runtime of less than ten minutes. This chiral separation is used to quantitate the four Beraprost sodium stereoisomers present in the bulk drug, tabletted formulations and in pharmacological and toxicological studies. The mobile phase variables that were found to have an effect on the stereoisomeric separation were studied and include: ionic strength, type and concentration of organic modifier, mobile phase pH and column temperature. Optimum stereoisomer separation was achieved on the Chiral AGP column. Calibration curves were linear for all four stereoisomers over the range of 0.024 to 4.04 μg/ml using fluorescence detection (correlation coefficients were greater than 0.999). A detection limit of 0.004 μg/ml was found for each stereoisomer. This assay has been used to determine the ratio of the four stereoisomers in the bulk drug as well as in the final formulated tablets.

INTRODUCTION

The separation of enantiomers has become an important area of analytical chemistry, especially in the pharmaceutical industry. The ability to separate and quantitate enantiomers in a drug composed of a racemic mixture is being addressed by the regulatory agencies of the United States, Japan and the European Economic Community [1–5]. Areas where a chiral separation must be used for a racemic mixture are: bulk drug stability, drug product stability, pharmacology, toxicology and pharmacokinetic studies. Without the development of a chiral separation, critical information about the drug would not be available.

A new class of prostaglandin derivatives was discovered in 1976 [prostacyclin (PGI_2)] [6]. PGI_2 was found to be an inhibitor of platelet aggregation and also had potent vasodilating properties [7]. These properties lead to investigating its use of atherosclerois [8], diabetes [9] and uremia [10]. The instability of PGI_2 in acidic or neutral conditions has been a major problem in studying this drug. The half-life of PGI_2 in an aqueous solution at pH 7.0 and 25°C is about 2 min [11]. Due to the instability of PGI_2, research has been ongoing in the search for an analogue that is more stable and yet maintains the same anti-platelet and vasodilating properties.

The compound used in this study, Beraprost sodium {Sodium (1*R**, 2*R**, 3*aS**, 8*bS**)-2,3,3*a*,8*b*-tetrahydro-2-hydroxy-1-[(*E*)-(3*S**,4*RS*)-3-hydroxy-4-methyl-1-octen-6-ynyl]-1H-cyclopenta[*b*]benzofuran-5-butyrate}, is a prostacyclin analogue that is significantly more stable in aqueous solutions yet has the same properties as those found for the original PGI_2 [12–16]. Beraprost sodium (Fig. 1) is composed of two diastereomers with each diastereomer containing a pair of enantiomers. The ability to separate and quantitate the isomers present in a racemic mixture is important in pharmaceutical products,

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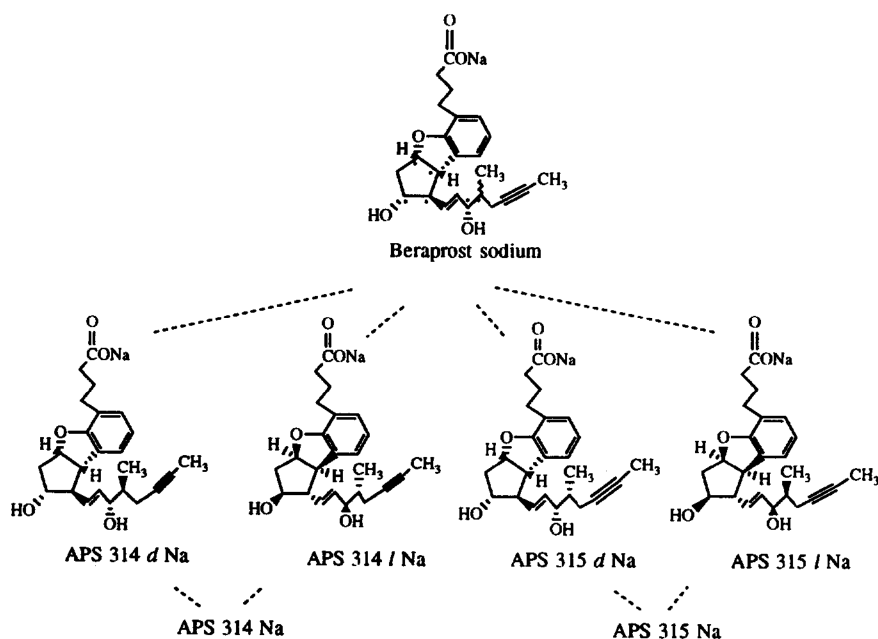

Fig. 1. Structure of Beraprost sodium and its four stereoisomers.

especially in terms of both bulk drug and formulation stability and purity [1].

A chiral separation of the four stereoisomers present in Beraprost sodium (BPS) has been accomplished. This separation uses a commercially available protein column (Chiral AGP) that is composed of an α_1-acid glycoprotein which is covalently bound to silica gel. The AGP column was introduced as a chiral selector in liquid chromatography in 1983 by Hermansson [17]. The molecular mass of AGP is 41 000 and the protein is comprised of polypeptide and carbohydrate moieties that account for approximately 55% and 45% of its mass, respectively. The isoelectric point in phosphate buffer has been found to be 2.7 [18].

AGP is an extremely stable protein that tolerates organic solvents and high temperatures, and a wide pH range without being denatured [19]. Direct chiral separations of racemic mixtures can be performed on the AGP column for diverse types of compounds including primary, secondary and tertiary amines, acids and non-protolytic compounds. The exact retention mechanism on an AGP column is not known. However, the column does show reversed-phase characteristics which allow many possibilities for changing enantioselectivity and retention through changes in mobile phase composition. Retention also appears to be due to a combination of hydrogen bonding, hydrophobic, electrostatic (coulombic) and charge transfer interactions [20,21].

The sample capacity of the AGP column has been investigated [21,22]. It was found that a 10-fold increase in the amount of metoprolol injected (0.5 to 5 nmol) resulted in a 10% decrease in retention with no effect on peak symmetry. The α value was unchanged over the range of sample studied. From this study, it was recommended that 3 to 5 nmol of analyte be injected into the analytical column.

The ruggedness (stability) of the Chiral AGP column has also been studied. A mobile phase consisting of 10.0 m*M* phosphate buffer, pH 7.0 with isopropanol (IPA)–water (6:94) was used for the separation of bupivacaine and mepivacaine as test compounds. Capacity factors of the test compounds were almost unaffected after the passage of approximately 40 000 column volumes (about 40 l) of mobile phase [19]. The AGP column also appears to be stable with high

concentrations of organic modifiers, buffers, and at elevated column temperatures without noticeable deterioration [19].

Not only have clean analytical samples been run on the Chiral AGP column, so have samples derived from biological matrices. The determination of enantiomers at low concentrations in plasma and urine has been accomplished [23,24]. The AGP column has been used for determining the enantiomers of atenolol, a β-receptor blocking agent, in human plasma at 1 ng/ml level for each enantiomer [25].

This paper will discuss the various mobile phase parameters that were studied and what affect each parameter had on the separation of the four stereoisomers present in Beraprost sodium. Detection limits and quantitation limits as well as calibration data will also be presented.

EXPERIMENTAL

Reagents and instrumentation

Beraprost sodium and the four stereoisomers (APS 314*d*, APS 314*l*, APS 315*d*, APS 315*l*) were obtained from Toray Industries (Kamakura, Japan). Acetonitrile, methanol and IPA (all HPLC-grade) were obtained from Burdick and Jackson (Muskegon, MI, USA). Phosphoric acid, sodium dibasic phosphate, and sodium hydroxide were obtained from Mallinckrodt (Paris, KY, USA). HPLC-grade water was obtained by passing deionized water through a Nanopure II water purification system (Barnstead, Dubuque, IA, USA). The instrumentation consisted of a Waters Model 600E system controller, Waters Model 600 solvent delivery system, Waters Model 700 Satellite WISP autosampler (Waters, Milford, MA, USA), a Linear Model LC-304 fluorescence detector (Linear Instruments, Reno, NV, USA), Waters Model 484 variable wavelength UV detector and Beckman PeakPro Chromatography data system (Beckman, Fullerton, CA, USA). The Chrom Tech (100 × 4.0 mm, 5 μm) Chiral AGP column was purchased from ASTEC (Whippany, NJ, USA).

Procedures

Standard analyte samples were weighed out in a humidity controlled glove box. (Beraprost sodium is a potent vasodilator and very hygroscopic, therefore care must be taken when handling dry samples of bulk drug.) HPLC-grade water was added to the volumetric flask before taking the standards out of the glove box. Standards were then diluted to volume with HPLC-grade water and mixed. Calibration data was obtained by making at least four measurements at each data point (peak area *vs.* amount of analyte injected). Flow-rates of 1.0 ml/min were used for all separations. Fluorescence detection performed using an excitation wavelength of 282 nm and an emission wavelength of 304 nm. Injection volumes of 100 μl were used.

RESULTS AND DISCUSSION

The mobile phase parameters that had a significant effect on the resolution of Beraprost sodium isomers on the Chiral AGP column are: type and concentration of organic modifier, ionic strength, mobile phase pH and column temperature. Each parameter was studied to evaluate their influence on the separation of the Beraprost sodium stereoisomers.

Organic modifier

The type and concentration of organic modifier had a profound affect on stereoisomer retention and resolution. Small changes in the concentration of methanol, IPA and acetonitrile produced large changes in stereoisomer retention. Stereoisomer separations performed using IPA showed better peak shapes than mobile phases containing methanol. Acetonitrile provided similar stereoisomer separations to the IPA mobile phases. However enantioselectivities and resolution were better with the IPA mobile phases. Small changes in CH_3CN concentration did not affect the separations nearly as much as did slight changes in the amount of IPA; a mobile phase containing 2% CH_3CN was equivalent to a mobile phase that contained 1% IPA. However, when a combination of CH_3CN–IPA were used, peak shape and resolution were superior when compared to mobile phases that contained only CH_3CN or IPA. Fig. 2 shows the effect of CH_3CN concentration on the stereoisomer retention while Fig. 3 compares the

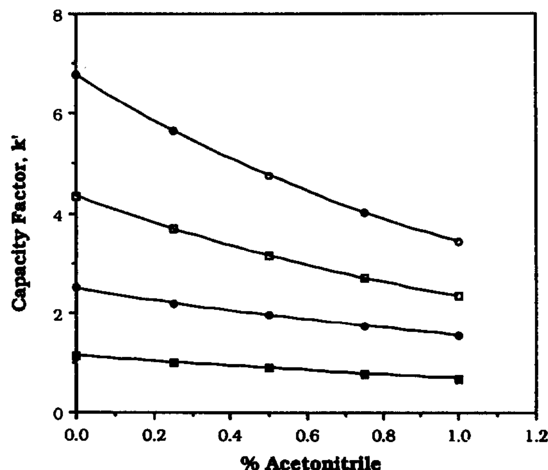

Fig. 2. Effect of acetonitrile concentration on the retention of Beraprost sodium stereoisomers on a Chiral AGP column. Mobile phase: 20.0 m*M* Na_2HPO_4, pH 7.0, CH_3CN–1.5% IPA–water. ■ = APS 314*l*; ● = APS 315*l*; □ = APS 314*d*; ○ = APS 315*d*.

separation of stereoisomers when two different concentration produced a significant difference in stereoisomer retention and in the time required for the separation. This shows that the organic modifier must be carefully measured to avoid changes in the stereoisomer separation.

Mobile phase ionic strength

Retention of each stereoisomer was directly proportional to mobile phase ionic strength (Fig. 4). As the concentration of buffer was increased, retention of the stereoisomers also increased. For example, when a mobile phase containing a 5.0 m*M* phosphate buffer at pH 7.0 was used, retention times for the stereoisomers were between 1 and 4 min. When the phosphate buffer concentration was increased to 60.0 m*M*, retention times for the stereoisomers increased to between 2 and 20 min. The increase in stereoisomer retention with increasing ionic strength may be attributed to salting out of the stereoisomers onto the stationary phase.

Mobile phase pH

Research has shown that mobile phase pH has a strong influence on the retention and enantioselectivity of basic, acidic and non-protolytic compounds [26]. In one study, anionic analytes

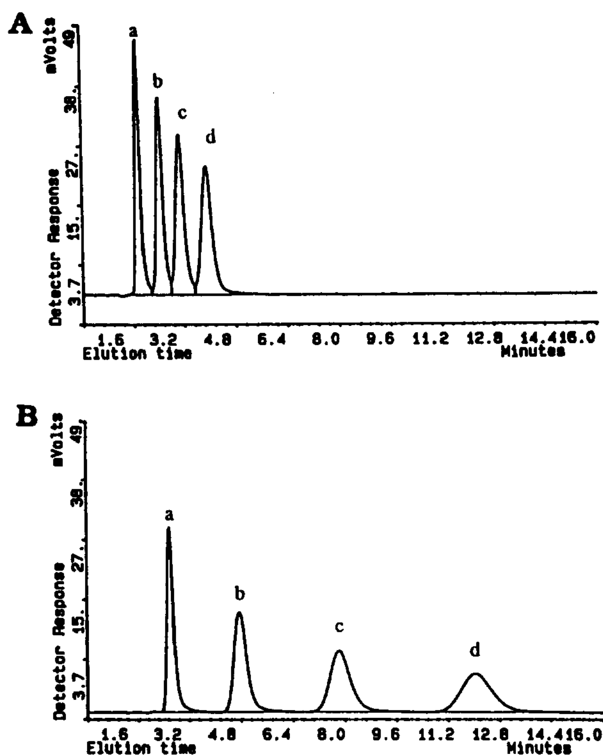

Fig. 3. Separation of Beraprost sodium stereoisomers on a Chiral AGP column using different concentrations of IPA. Mobile phase: 20.0 m*M* Na_2HPO_4, pH 7.0–0.5% CH_3CN–water (v/v/v), (A) 1.0% (v/v) IPA added to the mobile phase, (B) 0.5% (v/v) added to the mobile phase, a = APS 314*l*, b = APS 315*l*, c = APS 314*d*, d = APS 315*d*.

showed increased retention with decreasing mobile phase pH [27]. However, this study covered a pH range of 6.1 to 7.0 and did not investigate what effect lower pH mobile phases would have on anionic analyte retention.

Similar results were found for the BPS stereoisomers (Fig. 5). As the mobile phase pH was decreased from 7.0 to 3.0, retention of the stereoisomers increased until about pH 4.0 and then decreased. Enantioselectivity also decreased over this pH range. The increase in stereoisomer retention from pH 7 to 4 can be explained as follows. As the pK_a of the stereoisomers is approached, the stereoisomers become more hydrophobic and interact more strongly with the hydrophobic stationary phase. The stereoisomers had the highest retention times just below their pK_a values where the

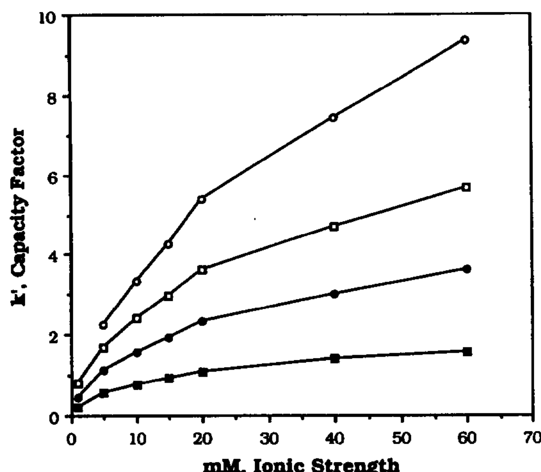

Fig. 4. Effect of ionic strength on stereoisomer retention: sodium phosphate buffer, pH 7.0, CH_3CN–IPA–water (0.5:1.0:98.5) mobile phase. ■ = APS 314*l*; ● = APS 315*l*; □ = APS 314*d*; ○ = APS 315*d*.

stereoisomers are mostly neutral. The decrease in stereoisomer retention below pH 4.0 can be explained by fewer ionic interactions and less hydrogen bonding taking place between the stereoisomers and the stationary phase as the stationary phase approaches its isoelectric point (pH 2.7).

Stereoisomer resolution as well as peak shape was best at a mobile phase pH of 7.0. Although the stereoisomers were still resolved at some lower mobile phase pH values, peak shape was unacceptable.

Column temperature

The effect of column temperature on the retention, enantioselectivities, and resolution of several basic drugs using an AGP column has been reported [26]. At higher column temperatures, enantioselectivity decreased while column efficiency increased. The increase in efficiency was attributed to faster transfer kinetics between the stationary phase and the stereoisomers.

Similar results were found for the four Beraprost sodium stereoisomers. Retention and selectivities of the stereoisomers decreased with increasing temperature, however, peak shape was improved (Fig. 6). Optimal peak shape was observed at a column temperature of 35°C.

Flow-rate

Several flow-rates were studied to determine if resolution would be affected. The flow-rates studied were 0.8, 0.9, 1.0 and 1.1 ml/min. Resolution was improved with lower flow-rates, however, peak tailing increased. A flow-rate of of 1.0 ml/min provided the best compromise between resolution and peak tailing.

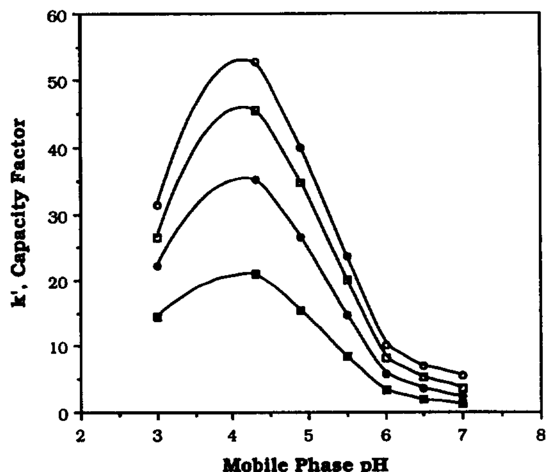

Fig. 5. Effect of mobile phase pH on stereoisomer retention: a 20.0 m*M* Na_2HPO_4, CH_3CN–IPA–water (0.5:1.0:98.5, v/v/v) mobile phase. ■ = APS 314*l*; ● = APS 315*l*; □ = APS 314*d*; ○ = APS 315*d*.

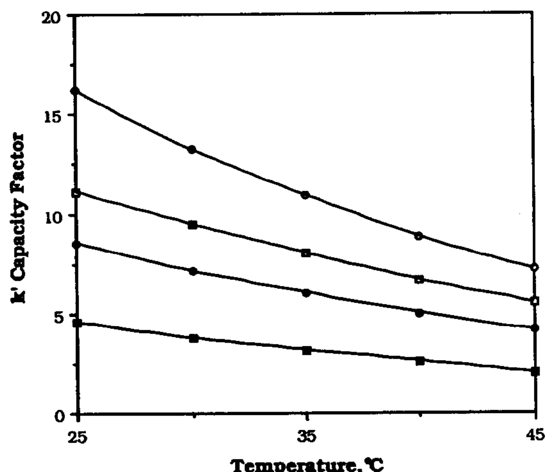

Fig. 6. Effect of column temperature on stereoisomer retention: a 20.0 m*M* Na_2HPO_4, CH_3CN–IPA–water (0.5:1.0:98.5, v/v/v) mobile phase. ■ = APS 314*l*; ● = APS 315*l*; □ = APS 314*d*; ○ = APS 315*d*.

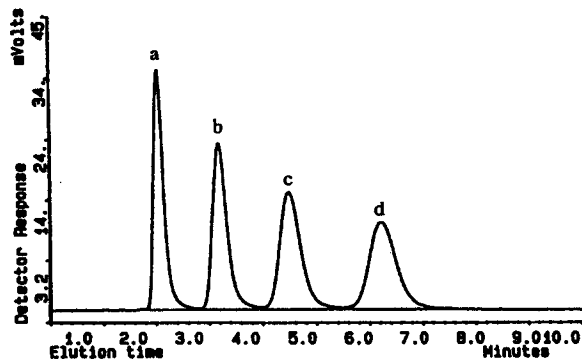

Fig. 7. Optimized separation of Beraprost sodium stereoisomers on the Chiral AGP column: a 20.0 m*M* Na_2HPO_4, CH_3CN–IPA–water (0.5:1.0:98.5, v/v/v) mobile phase; column temperature; 35°C. a = APS 314*l*, b = APS 315*l*, c = APS 314*d*, d = APS 315*d*.

Separation

The optimized separation for the Beraprost sodium stereoisomers is shown in Fig. 7. The mobile phase was composed of Na_2HPO_4 (20.0 m*M*, pH 7.0 adjusted with NaOH) and a solvent composition of CH_3CN–IPA–water (0.5:1.0:98.5, v/v/v). A flow-rate of 1.0 ml/min and a column temperature of 35°C were used. The four stereoisomers were baseline resolved with a runtime of ten min.

TABLE I

METHOD PRECISION AT DIFFERENT CONCENTRATIONS OF BERAPROST SODIUM

Determined using four injections at each concentration.

μg/ml	R.S.D. (%)			
	314*l*	315*l*	314*d*	315*d*
4.04	0.42	0.34	0.23	0.32
3.06	0.22	0.19	0.22	0.26
1.97	0.25	0.56	0.70	0.82
0.985	0.45	0.21	0.75	0.20
0.747	0.74	0.67	0.99	1.10
0.245	1.13	0.73	2.09	5.03
0.202	1.16	1.82	2.03	4.34
0.149	1.82	0.89	2.40	2.82
0.118	1.85	0.38	1.40	1.30
0.049	4.65	3.81	6.97	3.49
0.024	4.97	10.31	4.30	5.48

Calibration curves and sample loading

Calibration curves were established over the range 0.024 μg/ml to 4.04 μg/ml. A minimum of four injections of each standard was performed. Intraday assay accuracy ranged from 97.4 to 101.9% with a precision of ±1.29%.

TABLE II

EFFECT OF SAMPLE LOADING ON PEAK TAILING AND RESOLUTION

BPS Na[a] (μg/ml)	Peak tailing[b]				Resolution[c]		
	314*l*	315*l*	314*d*	315*d*	*l*/*l*	*l*/*d*	*d*/*d*
45.5	1.71	1.74	1.81	1.95	1.96	1.64	1.71
40.9	1.68	1.69	1.77	1.87	2.03	1.70	1.77
36.4	1.65	1.64	1.70	1.81	2.11	1.77	1.86
29.1	1.61	1.58	1.61	1.67	2.22	1.87	1.98
23.7	1.57	1.49	1.54	1.59	2.30	1.97	2.09
18.2	1.53	1.45	1.46	1.47	2.41	2.07	2.20
14.6	1.50	1.41	1.40	1.38	2.47	2.13	2.26
9.10	1.45	1.33	1.30	1.28	2.61	2.27	2.40
4.6	1.42	1.29	1.27	1.22	2.73	2.36	2.49
0.45	1.38	1.22	1.15	1.14	2.85	2.48	2.51
0.05	1.29	1.11	0.98	1.06	2.93	2.38	2.31

[a] BPS Na = Beraprost sodium.
[b] Calculated using USP XXII peak tailing method.
[c] Calculated using USP XXII resolution method.

Interday assay accuracy ranged from 97.9 to 101.4% with a precision of ±2.15% (Table I). Correlation coefficients of greater than 0.999 and detection limits of 0.004 μg/ml with a signal-to-noise ratio of 3:1 were found for each stereoisomer. This chromatographic system has been shown to be extremely rugged and reliable with over 1000 injections being done with minimal changes in retention or resolution.

The amount of sample injected onto the AGP stationary phase influenced both peak tailing and resolution (Table II). Peak tailing and resolution were better when smaller amounts of analyte was injected. Therefore, the AGP column is sensitive to the amount of injected analyte. This should be taken into account when determining how much analyte can be chromatographed.

Uses for the chiral HPLC assay

Beraprost sodium is by definition a racemic compound that contains four stereoisomers. The bulk drug and formulated tablets, as part of the release specifications, are assayed using this chiral HPLC method to insure that the ratio of stereoisomers is 1:1:1:1. This chiral HPLC assay is also used to determine the stereoisomer ratio for current and proposed bulk drug and formulated tablet stability studies.

ACKNOWLEDGEMENTS

Part of this work has been presented at *16th International Symposium on Column Liquid Chromatography (HPLC '92), Baltimore, MD, June 1992*, the *Midwest Regional American Chemical Society Meeting, Lawrence, KS, November 1992*, and the *7th Annual Meeting and Exposition, American Association of Pharmaceutical Scientists, San Antonio, TX, November, 1992.*

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